Bacteriology of Fish and Shellfish

P. K. SURENDRAN

Microorganisms are living creatures which are microscopic in size (i.e., they can be seen only through a microscope). They are relatively simple, often single-celled in structure.

Strictly speaking, microbiology began when people learned to grind lenses from pieces of glass and to combine them to produce magnifications, great enough to enable them to see microbes. Antony Van Leewenhock (1632-1723) was the first to report, with accurate descriptions and drawings, his observations under the microscope. He is considered the father of microscope. He made more than 250 microscopes, some of which could magnify upto 300 times. However, it was the great Louis Pasteur who founded the Science of Microbiology as we know it today.

Bacteria

Bacteria are single-celled (unicellular) microorganisms widely distributed in nature.

Of the five kingdoms to which the whole living things are grouped, bacteria belong to the lowest kingdom - the Prokaryota. The most outstanding characteristic of the organisms belonging to this kingdom is that their genetic material (nuclear material) is not bound by a membrane.

Morphology of bacteria

1. Shape

Bacteria occur in any one of three fundamental shapes, namely:

1. Spherical or oval shaped - called coccus (plural - cocci)
2. Cylindrical or rod shaped - called bacillus (plural - bacilli)
3. Spiral or curved rods.

The rigid cell wall of the bacteria is responsible for the shape of the bacteria.

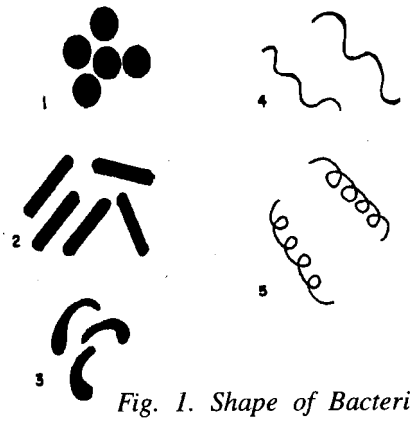

Fig. 1. Shape of Bacteria

(1) Spherical shaped - Coccus, (2) Rod shaped - Bacillus
(3) Spiral or curved rods - Vibrios (4) Rigid spiral form - Spirilla
(5) Flexuous spiral forms - Spirochaetes
(4 and 5 are modified spiral forms)

2. *Arrangement*

Most bacteria show characteristic arrangement of cells. These arrangements are the results of cell divisions in various planes. For example, spherical bacteria (cocci) can divide in one, two or three planes. Division in one plane gives rise to single cells, pairs or chains. Division in two planes produce clusters and division in three planes produces packets.

Cocci arranged in pairs are called diplococci; those in chains are called streptococci and those arranged in fours, tetrads. Groups of eight are called sarcina and grape-like bunches are called staphylococci. These names refer only to the cell arrangements and not to their scientific names.

Rod-shaped bacteria (bacilli) also show characteristic cellular arrangements, as pairs or chains. Some cells are arranged at angles to each other. But in many cases, there may not be any characteristic arrangement of cells.

3. *Size of bacteria*

Bacteria are microscopic in size. Their usual size range is so small that they are measured in microns. One micron is 1/1000th of a millimeter. The spherical is measured by its diameter and the rod or spiral shaped bacteria by their length and diameter.

Majority of the spherical bacteria measure about 0.5 micron in diameter. Rod shaped bacteria are in the size range of 1 to 5 micron in length and 0.5 to 1 micron in diameter. Sizes of some common bacteria are given below.

Escherichia coli - 1 to 3 micron in length x 0.5 micron in dia.

Bacillus - 2 to 5 micron in length x 0.8 micron in dia.

Staphylococcus aureus - 0.8 to 1 micron in dia.

4. *Bacterial cell*

Structure of a typical bacterial cell is given in figure 2. The cell consists of a compound membrane enclosing the protoplasm. The protoplasm is the living material. It is a thick viscous fluid-like jelly. It is colourless and transparent. The body of protoplasm in the bacterial cell is called the protoplast. In the protoplasm, nuclear bodies, ribosomes, mesosomes, polysaccharides, lipids and granules are seen.

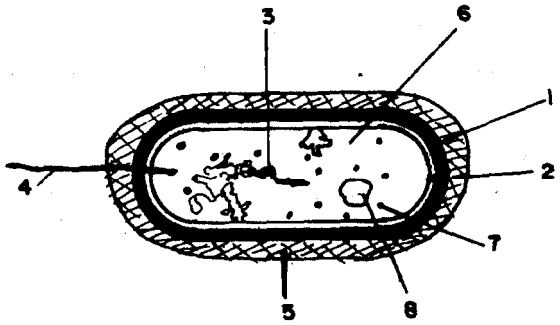

Fig. 2. Structure of Bacterial Cell

(1) Cell wall, (2) Cytoplasmic membrane, (3) Nuclear bodies, (4) Flagella, (5) Capsule, (6) Protoplasm (7) Granules, (8) Vacuole

Cell wall

The bacterial cell is enclosed in a rigid cell wall. The cell wall gives the shape to the bacterial cell.

Cytoplasmic membrane

Cytoplasmic membrane or the plasma membrane is the compound membrane enclosing the protoplast. It is very thin and semi-permeable. It is made up of lipids and proteins. It regulates the movement of water and metabolites into and out of the cell. The Gram staining and acid fast staining reactions of the bacteria are due to the cytoplasmic membrane.

Nuclear bodies

The bacterial cell does not have a well differentiated nucleus. The nuclear material is the genetic material of bacteria. It is a very long molecule of DNA (Deoxyribonucleic acid).

Flagella

Movement of bacteria - bacterial motility - is due to organs of locomotion, called flagella (singular - flagellum). Flagella are filamentous appendages. They originate in the protoplasm and extend out through the cell wall. They are thin and long. It is due to the propulsive movement of flagella that bacterial cell moves. Flagella are so thin that they cannot be seen by ordinary microscope. By special staining technique they are rendered thick and hence visible under light microscope. Otherwise they are seen only through electron microscope. Some bacteria may have one or more flagella on one end of the cell or on both ends. They are called polar flagellated. Others may have flagella all over the cell. They are called peritrichous flagellated.

Capsule and slime

Certain bacteria have a relatively thick gel-like covering layer outside their cell wall. This covering layer is called capsule. In some bacteria, this slimy gelatinous substance may not remain in firm contact with the cell wall, but becomes loose and disperses into the growth medium. In

such cases, it is called loose or free slime. Chemically, capsules are composed of protein and carbohydrate complex, while free slime is made of polysaccharides.

In the protoplasm, vacuoles and certain granules, which may be lipids, polysaccharides, polyphosphates etc. are also seen.

5. Bacterial reproduction

Bacteria multiply by simple division, called binary fission. When the bacteria are grown in a suitable medium under favourable conditions, the bacterial cell grows in size. Usually, the cell elongates to twice its, original length (or diameter in the case of coccus). The protoplast divides into two approximately equal parts by the formation of a transverse septum from the cell wall. In some species of bacteria, this cell wall septum splits into two. Thus two daughter cells are formed immediately. In others, the cell walls of the daughter cells remain continuous for some time. Such organisms grow in pairs, clusters, chains or filaments. In favourable conditions, the growth and division are repeated very rapidly, for example every half an hour or so. As a result, one individual cell may give rise to thousands of millions of new cells in one day. Actually, this does not happen in nature, mainly because of depletion of food and accumulation of toxic metabolites in growth media.

The time taken by a bacterial cell to grow and reproduce into two cells, i.e., the time between two successive cell divisions, is called a generation time. For most of the common bacteria, the generation time under favourable conditions is half an hour to one hour. However, for certain bacteria, this may be many hours or days. Examples of generation time :

Escherichia coli : 20-30 min.
Tubercle bacilli : 20 hrs. or more
Leprosy bacilli : 20 days or more

6. Growth phases of bacteria in a culture

When bacteria are inoculated into suitable culture media, under favourable conditions, most bacteria multiply at a very rapid rate. While

growing in the media, they produce pronounced changes in the culture media.

Under favourable conditions, a single cell of bacterium, for example, *E. coli,* divides into two in about 30 min. At this rate, a single cell of *E. coli* can produce 1000 million new cells by about 12 hours. But, this rate of multiplication is not continued indefinitely. As stated earlier, depletion of nutrients and accumulation of toxic waste products do not allow indefinite multiplication. Also, many cells die as the culture gets aged.

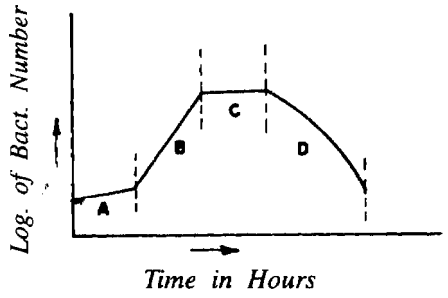

Fig. 3. Growth curve of Bacteria

(a) Lag Phase, (b) Logarithmic Growth Phase (c) Stationary Phase
(d) Death Phase

When a bacterium is inoculated into a suitable culture medium, the bacterial growth and reproduction do not take place in a regular manner. If we plot a graph, taking the logarithm of the bacterial population on the X-axis and the time interval on the Y-axis the figure shown (Fig. 3) will be obtained. This figure is called the growth curve or growth phase of bacteria in a culture. Four important growth phases are recognised in the curve.

a) The lag phase

During this phase, there is no increase in the number of bacterial cells. The bacterial cells adjust themselves to the medium and start growing in size. But they do not multiply. So, the graph shows only a horizontal line (part 'A').

b) The logarithmic growth phase (or the exponential growth phase)

Growth is maximum during this phase. Bacteria multiply at regular intervals and the rate of multiplication is constant. Therefore, the generation time is the same throughout this phase.

c) The stationary phase

During this phase, the number of living cells remains constant. The number of cells dying will be equal to the number of newly generated cells; i.e, the death rate equals the rate of increase.

d) The death phase (Decline phase)

More cells die during this period compared with new cells that are produced. So, there will be a steady decline in the number of living cells.

Under ideal conditions, the above pattern is observed in bacterial growth. But in nature, ideal conditions are not always present and hence, variations in the pattern of growth phases are to be expected.

7. Spore formation

Usually when conditions become unfavourable, the bacterial cell dies out. But in the case of certain Gram positive bacteria, a resting phase called 'spore' is formed. This resting phase is also called endospore. Endospores are bodies produced within the cells of bacteria. They are more resistant to unfavourable environmental conditions like heat, cold, desiccation, osmosis and chemicals. Spore formation is limited almost entirely to two genera of rod shaped bacteria, namely *Bacillus* and *Clostridium.* Sporulation is not a method of multiplication, but only a method of survival.

8. Staining of bacteria

Morphological shapes of bacteria are seen under a microscope. For clear vision, bacterial cell is given a suitable colour, using certain dyes (stains). This process is called staining. When a single dye is used to stain the bacterial cell, the process is called simple staining. Commonly

used simple stains are dilute solutions of methylene blue, crystal violet or carbol fuchsin.

When two or more dyes are used to stain bacterial cells, it is called differential staining. Such staining can bring about differentiation between different bacterial groups or different components of bacterial cell. Most commonly used differential staining process is Gram staining.

Gram staining

This differential staining process was devised by Christian Gram. Crystal violet (a para rosaniline dye) and safranine are the two stains used in this staining process. A dilute solution of crystal violet and ammonium oxalate is used as the primary stain to act on the bacterial film for a definite time (usually one minute). After washing away the stain with water, a dilute solution of iodine is added on the film and allowed to act for one minute. Next, this slide is destained by washing with alcohol. Finally the safranine is added as counterstain and allowed to act for another one minute, washed with water, dried and observed under microscope.

Some bacteria retain the violet stain even after treatment with decolourising agents like alcohol. They are called Gram positive (G +ve). Other microorganisms which fail to retain crystal violet, but take the counterstain (safranine) and appear red are called Gram negative (G -ve). Gram staining is very important in bacteriology. Based on Gram staining, bacteria are divided into two groups - Gram positive and Gram negative. Some examples are given below.

Gram + ve - *Bacillus, Staphylococcus*
Gram - ve - *Escherichia coli, Salmonella, Vibrio*

9. Effect of environment on bacterial growth

Bacterial growth is markedly influenced by many factors such as temperature, pH of the growth medium and the nature of the gaseous environment.

a Temperature

Each bacterial species has a range of temperature in which it has optimum growth. Also, there is a minimum temperature below which the bacterial growth does not take place and a maximum temperature above which bacteria cannot grow. The optimum growth temperature is the most favourable temperature for growth.

Depending on their temperature preferences, bacteria are broadly divided into three groups.

(i) Psychrophilic bacteria

They are cold loving bacteria. They grow usually between temperature ranges of 0-20°C, the optimum being 15°C. This group includes most of the bacteria causing spoilage of refrigerated or iced foods.

eg : *Pseudomonas, Alteromonas, Moraxella*

In actual practice, truly psychrophilic bacteria are not usually encountered. Those cold loving bacteria which we come across have a growth temperature range of 0-35°C and they are hence called psychrotrophic bacteria.

(ii) Mesophilic bacteria

Majority of the bacterial species belong to this group. They grow within the temperature range of 20-45°C with an optimum of 30-37°C. Most of the pathogens belong to this group.

eg: *Salmonella, Vibrio, Streptococcus*

(iii) Thermophilic bacteria

Bacteria which grow best at higher temperatures come under this group. Their growth temperature range is 45-70°C, the optimum temperature being 55°C. Bacteria belonging to this group are quite rare. Such bacteria are found in natural hot springs. Certain bacteria causing spoilage of canned foods belong to this group.

eg: *Bacillus coagulans, Bacillus stearothermophilus*

b. Oxygen/air

Depending on the requirement of oxygen/air for growth, bacteria are divided into 4 groups.

(i) Aerobic bacteria

Bacteria requiring the presence of free oxygen (or air) for their growth are called aerobic bacteria or aerobes.

eg: *Staphylococcus aureus*

(ii) Anaerobic bacteria

Bacteria which can grow only in the absence of free oxygen are called anaerobic bacteria or anaerobes.

eg: *Clostridium welchii*

(iii) Facultatively anaerobic bacteria

Bacteria growing in the presence and absence of free oxygen are called facultatively anaerobic bacteria.

eg: *Listeria*

(iv) Microaerophilic bacteria

Bacteria under this group grow in the presence of very little free oxygen.

eg: *Lactobacillus, Streptococcus*

10. Bacteria in fish spoilage

a. Native bacterial flora of fishes

The flesh and body fluids of live healthy fish are generally free from bacteria, i.e., sterile. But, even when the fish are alive, they harbour bacteria, mainly on three sites of their body - the slime on the skin surface, the gill tissue and the intestine. Bacteria, which are naturally present on fish are called the native bacterial flora of fish. The population and nature of such flora depend on the waters from where the fish are caught, i.e., whether seawater, brackishwater or freshwater.

Seawater has more dissolved salts, i.e., a higher salinity, than both the brackish and freshwater (See Table 1).

Table 1. *Salinity of different waters*

Water	Salinity range
Seawater	30 to 36 ppt*
Brackishwater	10 to 28 ppt
Freshwater	0.2 to 0.1 ppt

* ppt = parts per thousand

Depending on the salinity and pollution range of waters, the bacterial flora of fishes vary from water to water. Generally the bacterial population of the fishes from tropical waters are in the following ranges (Tables 2-4).

Table 2. *Bacterial population of marine fishes from tropical waters*

Sample	Oil sardine (*Sardinella longiceps*)	Indian mackerel (*Rastrelliger kanagurta*)
1. Skin with slime/cm^2	$10^3 - 10^7$	$10^4 - 10^6$
2. Gills/g	$10^5 - 10^8$	$10^4 - 10^9$
3. Intestine with contents/g	$10^5 - 10^9$	$10^5 - 10^8$

Table 3. *Bacterial population of brackishwater fishes*

Sample	Pearl spot (*Etroplus suratensis*)	Milk fish (*Chanos chanos*)
1. Skin with slime/cm^2	$10^3 - 10^4$	$10^3 - 10^5$
2. Gills/g	$10^4 - 10^8$	$10^5 - 10^8$
3. Intestine with contents/g	$10^5 - 10^8$	$10^6 - 10^8$

Table 4. *Bacterial population of freshwater fishes*

Sample	Rohu (*Labeo rohita*)	Mrigal (*Cirrhinus mrigala*)
1. Skin with slime/cm^2	$10^4 - 10^5$	$10^4 - 10^5$
2. Gills/g	$10^4 - 10^6$	$10^5 - 10^7$
3. Intestine with contents/g	$10^5 - 10^7$	$10^4 - 10^6$

Generally, the bacterial populations on the skin surface are the least, while bacterial counts in the intestine are the highest. Bacterial counts in the gill tissue are more or less between the bacterial counts of skin surface and the intestine. Also, bacterial populations exhibit seasonal variations. During warmer months, the counts are higher while during colder seasons, the bacterial counts are lower.

Total bacterial populations of some common prawns are given in Table 5. Generally, cultured prawns (*P. monodon* and *M. rosenbergii*) harbour higher bacterial load.

Table 5. *Bacterial population on freshly caught prawns*

Prawn	Total bacterial count/g. muscle
1. Naran *(Penaeus indicus)*	$10^3 - 10^5$
2. Kazhanthan *(Metapenaeus affinis)*	$10^2 - 10^6$
3. Poovalan *(M. dobsoni)*	$10^3 - 10^6$
4. Cultured Tiger prawn *(P. monodon)*	$10^4 - 10^6$
5. Cultured giant freshwater prawn *(Macrobrachium rosenbergii)*	$10^4 - 10^6$

Qualitatively, the composition of bacterial flora of the fishes from the three waters differ considerably. Majority of the bacterial flora of marine fishes are Gram negative, non-spore forming (asporogenous) rods or cocci. Bacterial flora of freshwater fishes are predominantly Gram

positive in nature. The microflora of the brackishwater fishes are evenly composed of both G +ves and G -ves.

Typical example of the generic composition of the bacterial flora of marine, brackishwater and freshwater fishes is given in Tables 6 - 8.

Table 6. *Microbial flora on the skin surface of oil sardine and Indian mackerel (both marine fishes) as % of total bacteria*

Bacterial genus	Oil sardine	Indian mackerel
a) **Gram negatives**	**85**	**81**
Vibrio	30	36
Pseudomonas	22	16
Moraxella	8	8
Acinetobacter	20	16
Flavobacteria	2	4
Aeromonas	1	1
Photobacterium	2	0
b) **Gram positives**	**11**	**13**
Arthrobacter	4	2
Micrococcus	6	6
Bacillus	1	5

Table 7. *Bacterial flora on the skin surface of pearl spot and milk fish (brackishwater fishes) as % of the total bacteria*

Bacterial genus	Pearl spot	Milk fish
a) **Gram negatives**	**57**	**60**
Pseudomonas	25	8
Alcaligenes	10	–
Flavobacteria	5	–
Moraxella	5	12
Vibrio	7	16
Acinetobacter	–	20
Coliforms	5	4

(Table 7 Continued)

b)	**Gram positives**	**36**	**34**
	Micrococcus	20	20
	Bacillus	5	4
	Arthrobacter	7	5
	Lactobacillus	2	5
	Streptococcus	2	–

Table 8. *Bacterial flora on the skin surface of Rohu and Mrigal (freshwater fishes) as % of the total bacteria*

	Bacterial genus	Rohu	Mrigal
a)	**Gram negatives**	**40**	**45**
	Pseudomonas	20	21
	Acinetobacter	10	19
	Coliforms	10	5
b)	**Gram positives**	**60**	**54**
	Micrococcus	60	30
	Bacillus	–	24

From these examples, it can be seen that in the case of marine fishes, not only is there a larger proportion of Gram negative bacteria, but also, there is a large number of bacterial types. But for freshwater fishes, the number of bacterial genera is quite limited.

Prawns also generally follow similar pattern in the distribution of bacterial genera. However, *Enterobacteriaceae* group of bacteria are present in brackish and freshwater prawns.

Table 9. *Bacterial flora of prawns as % of the total flora*

	Bacterial genus	Naran (marine) (*P. indicus*)	Tiger prawns (brackishwater) (*P. monodon*)
a)	**Gram negatives**	**86**	**61**
	Vibrio	5	16

(Table 9 Continued)

Pseudomonas	12	8
Moraxella	38	0
Acinetobacter	24	0
Aeromonas	2	10
Enterobacteriaceae	0	16
Others	5	11
b) **Gram positives**	**14**	**32**
Arthrobacter	12	0
Micrococcus	2	20
Bacillus	0	12

11. Spoilage of fish

Spoilage of fish, post mortem, is mainly due to (1) oxidation (2) autolysis and (3) bacteria. The major cause of spoilage of fish is bacteria, particularly in the case of marine fishes.

The flesh and body fluids of newly caught fish are free from bacteria (except when the fish has bacterial disease). The bacteria present on skin, adhering slime, in gills and intestine are normally saprophytic.

Once the fish is dead, these bacteria invade the fish tissue. There are three main routes for this attack.

1. From the gills into the flesh through the vascular (circulatory) system.
2. Through the skin by penetration.
3. Through the peritoneal lining (from the intestinal cavity).

Invasion of bacteria through the first and second routes is faster. Entry through the peritoneal lining can take place only after perforation of stomach and intestinal walls, which normally takes longer time.

The fish muscle contains 15 to 18% protein. Bacteria attack the protein and break it down to peptides and amino acids. Initially, bacteria live and multiply in the fish tissue, utilising the low molecular weight compounds like carbohydrates and amino acids present in small quantities in the muscle.

Due to post mortem enzymic breakdown of the macromolecules in the muscle also, enough low molecular weight compounds are formed in the muscle, which serve as the food of bacteria. Subsequently, bacteria elaborate proteolytic enzymes, which break down proteins to peptone, polypeptides, lower peptides and finally to amino acids. Amino acids will be metabolised by bacteria, in different ways, leading to the production of odoriferous and foul smelling compounds like ammonia, hydrogen sulphide, mercaptans, indole, amines and organic acids.

When left in our ambient temperature, which is usually 28±4°C tropical fishes get spoiled within 6 to 12 hours, depending on their size. In order to prevent such spoilage, many methods are in practice. Drying, icing, freezing, canning and use of chemicals are some of the usual methods. The basic principle involved in these methods of preservation of fish is to control the activities of the microorganisms.

a. Drying

Bacteria need water for their growth and multiplication. A minimum level of available water should be present in the medium or substance where the bacteria grow. Such available water in foods or other substances is described by the term water activity (a_w). Below a minimum level of water activity, microorganisms cannot grow. Most of the fish spoiling bacteria do not grow below a_w of 0.91. When we dry fish, water is removed from the fish muscle to an a_w of 0.9 or below so that bacterial action is completely prevented.

b. Icing

Icing is the most prevalent method of preserving fish. Ideal icing involves packing crushed ice and fish in layers in insulated boxes, in the fish to ice ratio of 1:1 (w/w). By this, the temperature of the fish is lowered to near 0.1°C in about 2-3 hours (the melting of the ice needs 80 calories of heat/g and this heat is removed from the fish in contact with ice and hence, the fish get cooled). This lowering of temperature brings about (1) arrest of almost all enzymatic changes, (2) killing of about 50-60% of the mesophilic bacteria and (3) slowing down of the activities and growth

of all other bacteria, which are cold-loving (psychrophilic) and cold-tolerant (psychrotrophic). As a combined effect of all these three factors, the spoilage of fish is delayed to a considerable length of time in ice. During iced storage of fish, there is an initial drop of bacterial count due to the death of the cold sensitive mesophiles. The surviving cold tolerant bacteria, however, get adapted to growth at low temperature. Consequently, there is a gradual increase in population, which takes about 6 to 8 days to reach a count of one million per gram or above. By that time, the fish has reached the stage of incipient spoilage.

Qualitatively, there is a selection of bacterial flora during iced storage of fish. Irrespective of the composition of the initial flora, the *Pseudomonas/ Alteromonas* group emerge as the predominant group of bacteria at the time of spoilage. This is because most of the psychrotrophic bacteria capable of causing spoilage belong to these genera.

A typical example of selection of bacterial flora of fish during iced storage is given in Table 10.

Table 10. *Pattern of change in the bacterial flora of oil sardine during iced storage*

Bacterial genus	% of the total flora			
	0 day	7 days	14days	21days
Pseudomonas/				
Alteromonas group	16	24	39	74
Moraxella	8	15	11	4
Acinetobacter	24	34	22	8
Vibrio	26	8	5	2
Flavobacterium/				
Cytophaga group	4	5	5	2
Micrococcus	8	6	7	4
Others including yeasts	14	8	11	6

In the case of tropical fishes, it is not the psychrophiles, but the psychrotrophs, which are the actual spoilers during iced storage. These psychrotrophs, whose population is very low in the fresh tropical fish, easily adapt to growth at low temperature during iced storage and grow very rapidly and spoil the fish. Further, psychrotrophs have a shorter generation time compared to psychrophiles.

c. Freezing

Freezing of fish is done at -40°C and the frozen fish is further stored at -18°C. During freezing, 80 to 90% of the Gram negative bacteria die out and the residual bacteria cannot grow in the temperature of frozen storage. So, during freezing preservation of fish, there is no bacterial spoilage. But, before cooking, the frozen fish has to be thawed. During the thawing process the residual bacteria, which are predominantly Gram positive, can cause spoilage of the thawed fish. Hence, frozen fish will have to be thawed within the shortest possible time.