QUANTITATIVE AND QUALITATIVE STUDIES ON THE BACTERIOLOGY OF FROZEN FISHES AND PRAWNS

THESIS

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by

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DECLARATION

I hereby declare that this thesis is a record of bonafide research carried out by me under the supervision of Dr. K.Gopakumar, my supervising teacher and it has not previously formed the basis for award of any degree, diploma, associateship, fellowship or other similar title or recognition to me, from this or any other University or Society.

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This is to certify that this thesis entitled "Quantitative and Qualitative Studies on the Bacteriology of frozen Fishes and Prawns" embodies the results of original work conducted by Mrs. Nirmala Thampuran under my supervision and guidance from 6-3-1982 to 11-2-1987. I further certify that no part of this thesis has previously been formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles of this or any other University or Society. She has also passed the Ph.D. qualifying examination of the Cochin University of Science and Technology in July, 1984.

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ABBREVIATIONS USED IN THE THESIS

g	gram
h	hour
I.U.	International unit
kg.	kilogram
L	litre
mg	milligrams
ml	millilitres
no.	number
rpm	revolutions per minute
ug	micrograms
n.saline	normal saline
ppm	parts per million
sq.cm.	square centimetre

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INTRODUCTION

1. INTRODUCTION

Fish, a cheap source of dietary protein, is fast gaining acceptance as the ultimate solution to the problem of malnutrition in many developing nations. Growing populations and greater affluence demand an expanded supply of high quality food. The marine resource alone, if properly organized can meet this demand.

Data on world catch of fish indicates that after progressing steadily for the past several years, the catch has come to a halt in 83-84 at a level of 76.42 million tons close to the catch of 76.35 million tons in 82-83 (Anon, 1984). If this trend persists, it is likely that the world will be faced with the problem of acute shortage of animal protein in the coming years.

Future world demand projections show that by the close of this century an anticipated world population of 6.1 billion will require additional 29 million tons of fish per year. The demand will be more in the developing countries which account for 90% of the population growth. Accordingly, such countries may have an extra demand of 5 million tons of fish by the year 2000 to maintain the present level of consumption.

The major concern in post harvest losses is the rejection due to spoilage - biochemical (post-mortem changes) microbial and autolytic changes. Autolytic enzymes aid this process. Changes in the bacterial flora and the degree to which these bacteria are able to grow will determine the microbial spoilage pattern of the product. This has relevance to the public health hazard when such fish is consumed. The loss due to spoilage points to the inability of the existing infrastructure to handle loads as it may happen during glut seasons.

The highly perishable nature of fish flesh has restricted the utilization of it to maximum possible extent. Icing, though very common, is applicable to short term preservation alone. For long term preservation of fish, Other methods like freezing or curing are sought. Freezing has become most accepted and popular practice, although it is at present solely export-oriented. Being cheap and very similar to fresh fish, frozen fish rates a greater consumer acceptability than the other products. By expanding the capacities and diverting the possibilities to the hitherto unexploited fishes, it is possible to maintin the supply.

Economy of freezing demands mass production and this necessitates in-depth studies to evolve products

of stabilised quality and longer shelf life. Consideration has to be given to microbiology of raw materials, the effect of various processing conditions on the microbial flora, including possible microbial contamination and growth during defrosting. Several microorganisms including some pathogenic types are capable of growth at low temperatures. The knowledge of the microbial profile is also important for setting up microbiological specifications as well as to arrest the growth of the bacteria.

Past workers have focussed their attention mainly on the food poisoning/indicator bacteria in fish and fishery products and relevant changes during different processing stages. There is a paucity of information on the bacterial flora of tropical fish and their changes during processing and storage. Eventhough there are excellent studies on the psychrotrophic bacteria of fishery origin and their role in spoilage of temperate or subtropical fishes, such data available for the tropical fish is rather scanty or limited.

This investigation has been undertaken with a view to identifying areas that should be considered in the production of bacteriologically safe fish and fishery products. It will also enable a full understanding of the bacteria responsible for spoilage

and the conditions which permit the growth of the pathogenic organisms in frozen fish. This thesis is aimed at collecting such basic information which are relevant and commercially useful to the processing and preservation of tropical fish.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Bacteriology of freshly caught marine fish and Shellfish

Majority of the surveys on the bacterial flora of fish conducted in the past, were prompted by the need to elucidate the nature of microorganisms responsible for their deterioration. (Reay and Shewan, 1949). Consequently much emphasis was given to geographical locations and difference in fish species. The literature concerning studies on the bacterial flora of cold water fish species is replete with examples (Shewan, 1962; Shewan and Hobbs, 1967; Shewan, 1977; Horsley, 1977; Liston, 1980). Such data on fish from tropical waters are scanty (Venkataraman and Sreenivasan, 1952, 1953; Shaikmahmud and Magar, 1956; Pawar and Magar, 1966; Jadhav and Magar, 1970; Karthiayani and Iyer, 1967; Anand and Setty, 1977; Surendran, 1980; Lima dos Santos, 1981).

The commensal bacterial flora is defined as those present on the gills, integuments and within the alimentary tract of the healthy fish (Horsley, 1977). While studying the bacterial flora of freshly caught fish, a number of factors have to be taken care of, some of which are intrinsic while others are extrinsic.

2.1.1 Factors influencing the bacterial flora

The technique employed to isolate the bacteria is found to have profound influence on the observed composition and this aspect has been elaborately reported by Horsley (1977).

Environmental factors markedly affected the bacterial flora (Shewan, 1962). According to him 'bacterial flora is a reflection of the environment from where fish is caught'. This is further supported by the observations of Wood (1953), Venkataraman and Sreenivasan (1952, 1953), Shewan (1971a) and Lee and Pfeifer (1975).

The mode of capture also has some significance in the quantitative nature of bacteria on the surface. It has been demonstrated that trawled fish carried 10-1000 times more bacteria on the skin surface than fish caught with a baited line (Horsley, 1977). This increase in bacterial count on trawled catch was presumed to be due to either contamination of the fish with sediment materials when the catch was dragged through the seabed or the release of intestinal contents of the fish when the trawl was hoisted up (Reay and Shewan, 1949; Colwell, 1962). The gills were apparently unaffected as the operculum protected gills from contamination by sediments (Shewan and Hobbs, 1967).

Season profoundly influenced the bacterial profile. This was observed by Liston (1956) and Georgala (1957a, b) for fish from temperate waters and Karthiayani and Iyer (1971) and Surendran (1980) for fish from tropical areas. The seasonal variation occurred not only on the total populations but in specific types also (Pradeep and Lakshmanaperumalsamy, 1984).

The micro-environment of fish integument, together with the condition prevailing in fish habitat may influence the bacterial flora (Horsley, 1977). The antibacterial activity of fish slime, the state of health of fish being analysed etc. have also to be accounted for while interpretting the bacterial flora of newly caught fish.

2.1.2 Bacterial flora of freshly caught fish

2.1.2.1 Quantitative aspects

The number of viable, aerobic, heterotrophic bacteria on the skin surface (per cm^2) of the fish from temperate zone at 20°C consisted of 10^2 - 10^5 bacteria, while gills and gut contents carried 10^3 - 10^7 and 10^3 - 10^8 bacteria per gram of tissue respectively (Shewan, 1962).

For tropical fish Indian Sardine, Karthiayani and Iyer (1967) recorded total bacterial counts of 10^5 - $10^7/cm^2$

for skin surface, 10^6 - 10^8/g for gills and 10^7 - 10^9/g for gut contents at 30°C. Simidu et al. (1969) reported counts of 10^4/g, 10^3 - 10^5/g and 10^3 - 10^7/g respectively for skin, gills and guts of the Japanese flat fish, Karieus sp. at 20°C. For Australian mullet etc. the skin surface carried 10^4 microorganisms/cm^2 (Gillespie and Macrae, 1975). Surendran and Gopakumar (1982) reported total bacterial count of 10^4 - 10^6/cm^2 of skin for Indian mackerel and 10^3 - 10^7/cm^2 of skin for sardine. For same species of fish Devaraju and Setty (1985) reported bacterial counts of 3.05×10^5 and 3.75×10^5/g (whole fish). For freshly caught demersal fishes Croaker (Johneops spp.) and pink perch (Nemipterus japonicus) Anand (1976) found the total bacterial counts to be the order of 0.3×10^5/g and 1.1×10^5/g while Devaraju and Setty (1985) reported counts of the order of 3.55×10^4/g and 3.4×10^4/g respectively.

Based on these data Shewan (1977) concluded that fish from warm waters frequently carry greater number of bacteria than cold/temperate water fish species. However Lima dos Santos (1981) after reviewing the available data on warm water fish species suggested that no comparison could be made between tropical and temperate/cold water fish species due to diversity of variable factors.

Shewan (1977) also observed that fish from cold areas carry more psychrophiles than the fish from tropical zones. This was based on the observation that fish samples from tropical zones yielded a higher bacterial count at higher temperature of incubation compared to the low temperature incubation.

2.1.2.2 Qualitative studies

Qualitative studies on fish bacteria and their involvement in spoilage from 1920 to 1945 have been reviewed by Reay and Shewan (1949). Later reviews on this subject are those of Shewan (1977) Horsley (1977) and Liston (1980). These summarize the best existing data available on the bacterial flora of freshly caught fish indicating the frequency of occurrence of various groups and their regional preferences.

It is obvious from these studies that the flora of skin and gill of fish is invariably characterized by the predominance of Gram-negative asporogenous rods, representing the genera Achromobacter, Vibrio, Pseudomonas Flavobacterium and Cytophaga. In addition to this small quantities of Micrococcus, Bacillus and Coryneforms are also encountered. A detailed list of various groups of bacteria occurring on fishes of different regions has been presented by Horsley (1977). This shows that Achromobacter or Pseudomonas have been frequently reported as the dominant

species in bulk of samples of fish from North sea, Norwegian, North Atlantic, Atlantic and in certain samples of North Pacific ocean. A preponderance of Vibrio spp. over 20% was noted by Karthiayani and Iyer (1967) for Indian sardine, Aiso et al. (1968) for jack mackerel, Trachurus spp. Simidu et al. (1969) for plaice, Karieus spp. Anand and Setty (1977) for some marine fishes and Surendran and Gopakumar (1982) for sardine and mackerel. For majority of the samples from temperate areas, Vibrio species were absent or present in low numbers only. A high percentage of Gram-positives such as Micrococcus and Bacillus were reported for Indian mackerel (Venkataraman and Sreenivasan, 1952, 1954) Australian mullet, Mullus spp. (Gillespie and Macrae, 1975) Australian barracuda, Sphyraena spp. whiting, Merlangius spp. mullet, Mullus spp. (Wood, 1953) and North Atlantic cod (Dyer, 1947). A high percentage of Micrococcus spp. was also reported for some marine fishes from Bombay coast (Shaikmahmud and Magar, 1956; Pawar and Magar, 1966; Jadhav and Magar, 1970). A dominance of Flavobacterium spp. was reported for fishes caught off Mangalore coast (Anand, 1976; Devaraju and Setty, 1985).

The bacterial flora of elasmobranchs present somewhat a different picture. The bacterial population of the Indian shark (Carcharius sp. Rafinesque) was

characterized by a preponderance of Gram-positive organisms, the most dominant ones being Coryneforms Micrococcus and Bacillus comprising 82.7% of the population. The Pseudomonas spp. absent in the fish, were numerous in the water samples (Venkataraman and Sreenivasan, 1955). However, Liston (1957) reported that the skin flora of North sea skate (Raja spp. L) was similar to that of teleosts with a predominance of Gram-negative flora.

The bacterial flora of the alimentary tract of fish has attracted the attention of researchers mainly because of their role in spoilage (Liston, 1957; Aiso et al., 1968; Sera and Kimata, 1972; Garcia-Tello and Zaleski, 1970; Karthiayani and Iyer, 1967; Surendran, 1980). According to Liston (1980), Pseudomonas, Moraxella, Acinetobacter, Aeromonas in addition to Clostridium spp. are frequently encountered in fish intestines. Enterobacteriaceae and flavobacteria are also present in low numbers in the alimentary tract of marine fish (Trust and Sparrow, 1974).

The bacterial genera present within the alimentary tract of fish may be reflection of the genera present in the food ingested (Shewan, 1961) and thus related to environment. This was supported by the work of Yoshimizu and Kimura (1976).

The observations of Aiso et al. (1968) and Sera and Kimata (1972) are quite contrary to the above finding. According to Sera and his colleagues, the flora of the digestive tract of fish may quite be independent of the food or the environments. They observed that flora of the digestive tract of fish was limited to a few groups as the conditions in the stomach was so severe as to exclude majority of the exotic types.

2.1.3 Bacteriology of shellfish

Limited information is available on the bacteriology of newly caught shellfish. Most of the data pertain to freshly landed shellfish only.

2.1.3.1 Quantitative aspects

The total bacterial count reported for shellfish varied from place to place (10^3 - 10^7/g muscle). Thus Green (1949a, b), Fieger (1950), Fieger et al. (1956) and Cobb and Vanderzant (1971) reported counts at 20°C, in the range of 10^4 - 10^7/g for shrimp from Gulf of Mexico, 10^3 - 10^7/g for Louisiana crab (Tobin and McCleskey, 1941), 10^3 - 10^6/g for scampi, Nephrops norvegicus (Walker et al., 1970; Cann et al., 1971), 10^3 - 10^6/g for some edible varieties of crab (Early, 1967), 10^4 - 10^7 for deep water shrimp, Pandalus borealis and 10^4 - 10^5/g for inshore shrimp Pandalus montagui and Crangon crangon (Cann, 1977).

The bacterial count reported for crustaceans from tropical area differed from cold water species, being mesophilic, requiring a higher temperature of incubation (Cann, 1977). Counts reported for prawns in India range from 10^4 - 10^7/g (Venkataraman et al., 1953; Pillai et al., 1961; Surendran, 1980), in Thailand, from 10^3 - 10^6/g (Cann, 1971) and in Northern Australia, 10^3 - 10^5/g (Ruello, 1974). Quantitatively the bacterial counts of prawns of different species were generally higher than those of teleost fishes (Bose, 1969).

2.1.3.2 Qualitative studies

Cann (1977) has summarized the results of the qualitative studies on the bacterial flora of shellfish from temperate and tropical waters. Major groups of bacteria comprising the flora of crustacean shellfish from temperate waters were generally found to be Micrococcus, Coryneforms, Moraxella - Acinetobacter (previously grouped as Achromobacter), Pseudomonas and to a lesser extent Flavobacterium/Cytophaga spp. and Bacillus. But wide variations in the relative proportions of bacterial genera were encountered in certain samples (Cann, 1977).

For tropical prawns Sreenivasan (1959) reported a predominantly micrococcal and corynebacterial flora whereas Bose (1969) reported a predominance of Gram-negative rods (90-95%) of which Vibrios dominated.

Wide variations were noted in the bacterial flora of crabs. Alford et al. (1942) reported Micrococcus to be predominating in the flora of Louisiana crab while for North sea crab (Early, 1967) and Pacific crab (Lee and Pfeifer, 1975), Achromobacter was predominant in both flesh and intestine. Venkateswaran et al. (1981) found a dominance of Micrococcus (52%) and Pseudomonas (43%) in the gut of edible crab from Porto Novo coast.

2.1.4 Presence of bacteria of public health significance

With the exception of molluscs, fish and shellfish from cold or temperate waters poses rarely as a problem in public health (Liston, 1980). This is because fish caught from open sea is virtually free from pathogens, excluding Vibrio parahaemolyticus and Clostridium bolulinum (Hobbs and Hodgkiss, 1982).

Usually members of Enterobacteriaceae are absent in fresh fish caught from open sea or unpolluted waters of coastal areas (Spencer and Georgala, 1958; Appleman et al., 1964; Shewan, 1971a), though fish from polluted water carried pathogens like Salmonella (Guelin, 1962; Shewan, 1971a).

Staphylococci were detected in fishes caught by commercial trawlers (Spencer and Georgala, 1958; Appleman et al., 1964). Since they were absent in freshly caught fish, they must be considered as contaminants. The presence of Vibrio parahaemolyticus in marine fish and sediments was reported by Kaneko and Colwell (1973), Koburger et al. (1975) and Thompson et al. (1976).

The tropical fish and prawn also showed the presence of bacteria such as Salmonella and coliform (Venkataraman and Sreenivasan, 1955; Rao and Gupta, 1978; Zuberi and Quadri, 1981). Vibrio parahaemolyticus was also found to be present in marine waters and fish (Bose and Chandrasekaran, 1976; Karunasagar and Mohan Kumar, 1980; Bandekar et al., 1982; Pradeep and Lakshmanaperumal Samy, 1984).

2.2 Response of bacteria to low temperature

Microorganisms capable of growth at low temperature have been known from very early times. Interest in low temperature microbiology increased tremendously during the past two decades possibly because of its involvement with spoilage reactions at low temperature and its role in organic cycles. The increase in interest prompted several workers to study this aspect and several

excellent reviews have come forth. The reviews of Ingram and Stocks (1959), Fennema and Powrie (1964), Farrell and Rose (1965), Farrell and Rose (1967), Morita (1975), Baross and Morita (1978) and Herbert and Bhakoo (1979) emphasizes the fundamental aspects of response of bacteria to low temperature. The applied aspect of it, namely the significance of these bacteria in foods, has been dealt with in the reviews of Borgstrom (1955), Michener and Eliott (1964) and Kraft and Rey (1979).

2.2.1 Psychrophiles, psychrotrophs and mesophiles

Traditionally microorganisms have been classed on the basis of cardinal temperatures into thermophiles, mesophiles and psychrophiles according to their ability to grow at high, medium and low temperatures respectively. Psychrophiles have been defined in various ways by various authors. According to Baross and Morita (1978), "Very few words in microbiology have been subjected to as many different definitions as the word psychrophile". Their ability to grow at 0°C, the maximum, optimum and minimum growth temperatures, growth rate at 0°C have all been used by various people to define this seemingly distinct physiological group of microorganisms. Detailed discussions on the definition of psychrophile has been attempted by Ingram and Stocks (1959) and Morita (1975).

The definition proposed by Morita (1975) is accepted by most of the workers in this field according to which psychrophile restricts its growth temperature range from 0°C or less to 20°C or less with a temperature optimum at 15°C or less. Some of the psychrophilic bacteria encountered in nature have their temperature maxima beyond 20°C while they show characteristic growth at 0°C and similarly many mesophilic types exhibit temperature minima at 0°C or less. The term 'psychrotroph' has been suggested to include all low temperature growing microorganisms not fitting Morita's description. They have been also referred by term 'facultative psychrophiles' while those fitting to Morita's description are termed 'obligate psychrophiles'.

The psychrotroph as defined by Eddy (1960) is an organism capable of growth at 5°C or below regardless of its maximum, optimum or minimum temperature. This group is quite significant since it includes many bacteria from refrigerated foods or seasonally variable environs. It should also be emphasized that much controversy still exists in the definition of psychrotroph and psychrophile.

2.2.2 Effect of temperature above freezing on microorganisms

2.2.2.1 Incidence of growth

The well-known effect of cold on microorganism is

to slow down and finally stop growth. When a substrate containing mixed microbial population is progressively cooled, the cells will continue to grow, but with an extended lag period, until the minimum temperature of growth is reached (Shewan, 1961). The minimum temperature of growth is the critical point in the growth of bacteria, yeast or mould. Ingram and Mackey (1976) defined minimum temperature of growth as the point where either the lag period or generation time becomes infinite.

The importance of minimum temperature of growth of microorganism has been elaborately discussed by Michener and Eliott (1964). The minimum temperature of growth of some of the pathogenic and food spoilage bacteria are enlisted in Table 1. The pathogenic/indicator bacteria which are also mostly mesophilic, possess a higher minimum growth temperature than the psychrotrophic spoilage bacteria. Various factors which affect the minimum growth temperature are described by Ingram and Mackey (1976).

Normally growth does not occur below the minimum temperature of growth. At about +5°C mesophiles generally ceased to grow, but some members of psychrophiles or psychrotrophs grew well beyond this

Table 1

Minimum growth temperature of spoilage, food poisoning and indicator bacteria

Bacterial strain	Source	Minimum temperature for growth °C
I. Psychrophilic spoilage bacteria[a]		
Vibrio anguillarum (NCMB-6)	Ulcerative lesion in plaice	-1
Pseudomonas fluorescens (NCMB-129)	Freshly caught cod	-4
Pseudomonas fragi (NCMB-320)	Iced cod (2 days)	-6.5
Pseudomonas putida (NCMB-406)	Freshly caught cod	-4
Pseudomonas sp.	Iced cod (12 days)	-4
Pseudomonas putrefaciens (NCMB-1735)	Unknown	-6
Pseudomonas putrefaciens (NCIB-8615)	Surface tainted butter	-2
Pseudomonas rubescens (NCIB-8767)	Cutting oil	-3
Moraxella like sp. species-78	Spoiling, prepacked cod fillets	-5

Table contd.

Table contd.

Bacterial strain	Source	Minimum temperature for growth °C
II. Indicator bacteria[b]		
Eacherichia coli	Crab meat	5
"	Oyster	4.6
Aerobacter aerogenes	Liquid egg	11.1
"	-	5
Coliform group	Oyster	4-6
Enterococcus	Oyster	4-6
III. Food poisoning bacteria[a]		
Staphylococcus aureus	-	6.7
Salmonella sp.	-	6.7
Vibrio parahaemolyticus	-	4

a - After Harrison and Church and cited by Shewan and Murray (1979)

b - After Michener and Eliott (1964)

temperature. Michener and Eliott (1964) cited many examples of incidence of growth of bacteria, yeast and mould in food stuffs as well as laboratory media at subzero temperatures. Thus growth is common at -5°C to -7°C and less or even rare below this temperature. A temperature of -12°C has been considered as the lowest limit of growth under all circumstances (Ingram and Mackey, 1976; Genigeorgis and Riemann, 1979).

The ability to tolerate reduced water activity (a_w) could also be a factor governing growth at subzero temperature. But information on this aspect is limited due to the practical problem of controlling a_w below zero (Ingram and Mackey, 1976). Larkin and Stokes (1968) studied the effect of a_w on the growth of some bacteria. It is seen that growth of organisms in the subzero zone i.e. from 0°C to -10°C would be expected only in species which having a minimum growth temperature possess the ability to tolerate reduced a_w or high solute concentration. Table 2 summarizes the minimum a_w for growth of some bacteria.

The loss of viability and injury occurring in bacteria held at non-freezing temperatures are of interest. The loss of viability was found to be associated with cold shock phenomenon. Early belief was that Gram-negative mesophilic types such as E. coli were more

Table 2

Minimal water activity (a_w) for multiplication of bacteria associated with meat and meat products

a_w	Bacteria
0.98	Clostridium bolulinum type C. Pseudomonas* Clostridium bolulinum
0.97	Clostridium bolulinum type E
0.96	Flavobacterium, Klebsiella, Lactobacillus* Proteus*, Pseudomonas*, Shigella
0.95	Alcaligenes, Bacillus, Citrobacter, C.botulinum type A & B and Clostridium perfringens, Enterobacter, Escherichia proteus, Pseudomonas, Salmonella, Serratia, Vibrio
0.94	Lactobacillus, Microbacterium, Pediococcus Streptococcus, Vibrio*
0.93	Lactobacillus*, Streptococcus
0.92	-
0.91	Corynebacterium, Staphylococcus (anaerobic) Streptococcus
0.90	Lactobacillus*, Micrococcus, Pediococcus, Vibrio
0.86	Staphylococcus (aerobic)
0.75	Halophilic bacteria

*some strains only

Compiled by Leistner and Rodel (1976)

susceptible to cold shock (Meynell, 1958). But, later work of Strange et al. (1961), Strange and Ness (1963), Jackson (1974) and Patterson and Jackson (1979a, b) showed that Gram-positives were also exposed to cold shock. However, cold shock was found to be more destructive to Gram-negative bacteria than Gram-positives (Katoh, 1969).

2.2.2.2 Biochemical acitivity

The biochemical activities of the microorganisms, grown at low temperature have been studied. Earlier workers (Hess, 1934; Kiser, 1944) observed that even though some bacteria grew at low temperature, many of activities of the organisms at its temperature optima were characteristically absent at low temperature. Ayres (1960) observed that the ability to produce the pigment by many pigmented bacterial strains were arrested at 5°C. Rey et al. (1969) provided a theoretical basis to this finding that freezing altered the adaptive enzyme forming system such as production of pigment while the constitutive enzyme forming system like proteases were unaffected. It is accepted by many that when growth occur at low temperature most of the biochemical activities are retained even though speed may be strikingly altered (Shewan, 1961).

2.2.3 Effect of freezing on bacteria

Freezing commonly begins at -1°C to -3°C depending on the nature of the substrate (Michener and Elliott, 1964). At temperature immediately below zero, substrate may contain enough water for growth of microorganisms (Ingram and Mackey, 1976). Inactivation occur during freezing and is followed by the inactivation frozen state. The physical factors implicated in the death of microorganisms resulting from freezing has been discussed (Mazur, 1960).

The early convention was that death of bacteria at freezing temperatures resulted mainly through mechanical action of extra cellular ice (Borgstrom, 1955). Above -10°C freezing occurred only externally and a cell which could make osmotic adjustments escaped death. On the otherhand, below -10°C the cell membrane failed to act as barrier to the proliferation of ice already formed outside the cell, resulting in intracellular ice formation. Thus the ultimate effect of freezing is thought to be due to ice formation and solute concentration, caused by damage to the semipermeable properties of the cell membrane, resulting in death or injury. (Ingram and Mackey, 1976). According to Tanaka and Yoh (1980), damage to deoxyribonucleic acid (DNA) was also involved.

2.2.3.1 Survival of bacteria during freezing and frozen storage

Although the survival rates during freezing and frozen storage are known for many bacterial species, the experimental procedures have been too diverse to permit any comparision. Among the four major groups of bacteria namely, Gram-positive and Gram-negative rods and cocci, the Gram-positive cocci were particularly resistant to freezing. They also survived much better than the Gram-positive rods or Gram-negative rods/cocci (Ingram and Mackey, 1976).

There is very little information on the effect of freezing temperatures on bacterial species naturally associated with marine environment. But,it should also be emphasized that elaborate studies have been conducted on the fate of pathogenic or indicator bacteria present in fish or fishery products.

Some of the studies which dealt with some common genera are those of Kiser (1943), Major et al. (1955), Postgate and Hunter (1961), Arpai (1962), Pogorzelska (1979) in addition to the very early works of Haines (1938) and Hess (1934).

2.2.3.2 Survival at low temperature of bacteria of public health significance

Pathogens do not differ very much from nonpathogens

in their survival at low temperature. There are numerous publications on the effect of low temperature on the growth and viability of pathogenic bacteria (Wieser and Osterud, 1945; Raj and Liston, 1961; Georgala and Hurst, 1963; Hall and Slade, 1980; Alcock, 1983). These reports clearly show that these bacteria can, to varying degrees, survive freezing and cold storage conditions.

Coliforms in general and salmonella species in particular are very sensitive to cold. Growth of salmonella is arrested at a temperature below 5.2°C (Matches and Liston, 1968). Freezing and storage at subzero temperatures caused considerable reduction in the bacterial population. Zawadski and Pogorzelska (1975) carried out studies on the effect of freezing and storage of Salmonella inoculated into minced meat. The numbers decreased by 10.7 to 15.2% after freezing, by 49.5 to 54.7% after storage at -23°C for 10 days and by 88.1% to 91.2% after 10 months. Dussault (1956), studying the survival of coliform bacteria on freezing observed that process such as freezing did not eliminate this organism though their number was drastically reduced. This was supported by the work of Gunderson and Rose (1948). But Raj and Liston (1961) while studying the survival of various pathogenic bacteria in brain heart infusion broth at -34.4°C found that after 393 days Salmonella typhimurium was virtually eliminated.

The survival of Salmonella and Escherichia coli in Pacific oysters after freezing was studied by Digirolamo et al. (1970). The study indicated that loss of viability was in two stages, a rapid initial stage followed by a gradual decline. Salmonella showed a survival of 1% after 48 hour.

Different Salmonella species differed in their susceptibility to cold. Hall and Slade (1980) studied the survival of five species of Salmonella in different substrates and found that the species differed in their survival, Salmonella typhimurium being most sensitive. Also survival varied in different substrate for both Salmonella and Staphylococcus. This was also supported by the finding of Raj and Liston (1961) who noted that seafood material afforded the best protection for this bacteria.

Survival of 6 strains of Salmonella stored in sterile chicken at -25.5°C was found to be proportional to the initial contamination and 20% of the initial population of Salmonella typhimurium survived 9 months of frozen storage (Gunderson and Rose, 1948). Also, Salmonella survived better at sub-freezing temperature than subzero temperature (Georgala and Hurst, 1963).

Recent studies of Mossel et al. (1981) showed that

members of the genus Salmonella seldom grew at 3°C or 4°C whereas majority of them grew slowly at 7°C. But according to population dynamics this growth was found to be insignificant. Accordingly refrigerated storage and distribution at 7°C posed little health risk.

E. coli proved to be less sensitive to freezing compared to Salmonella (Raj and Liston, 1961; Dussault, 1956; Digirolamo et al., 1970). But it showed wide variation in the survival.

Staphylococcus was also sensitive to freezing, though it showed better survival compared to Salmonella (Raj and Liston, 1961; Hall and Slade, 1980). Kraft and Rey (1979) observed that Staphylococcus, even though it survived better during freezing, was drastically reduced during frozen storage. Also, the cells subjected to freezing, on resuming normal growth, possessed the ability to produce the enterotoxin (Hall and Slade, 1980). The organism grew at a temperature between 6.7°C and 45.4°C and was capable of producing toxin at low temperature (Angelloti et al., 1961). A faster death was observed at -11°C than -30°C (Woodburn and Strong, 1960).

Temperature is an important factor affecting the survival of Vibrio parahaemolyticus. Most of the strains appeared to be sensitive to refrigeration and freezing

temperature (Baross and Liston, 1970; Matches et al., 1971; Beuchat, 1973). Compared to low freezing temperature, better survival was noted at higher temperatures of storage (Vanderzant and Nickelson, 1972; Chandrasekaran et al., 1985). Different strains differed in the susceptibility to cold and destruction occurred more rapidly during the first 24 hours. Studies on the relationship between low temperature growth and other parameters such as salt concentration or water activity (Beuchat, 1973) provided some basis for the survival of this mesophilic organism at very low temperatures. Johnson and Liston (1973) found that considerable numbers could survive at 6 - 8°C for even up to 130 days.

In contrast to these organisms already mentioned, enterococcus group possessed exceptionally high resistance to freezing and cold storage (Raj and Liston, 1961; Raj and Liston, 1963; Lekshmy, 1964).

2.2.4 Factors affecting survival during freezing

The survival of a bacterial population during freezing was found to be influenced by a number of factors.

The effect of initial cell concentration on the survival of bacteria was studied by Major et al. (1955) who observed that Gram-negative bacteria such as Escherichia coli, Salmonella gallinarum, Serratia

marcescens and Pseudomonas aeruginosa manifested a percentage survival which varied with initial cell concentration. Some other groups such as Micrococcus pyogenes and Bacillus pumilus also manifested a percentage survival though it was not very pronounced. On the otherhand for Lactobacillus sp. Chromobacterium sp. and Bacillus coagulans, survival was independent of initial cell number and found to be a constant.

Presence of salts markedly affected the survival during freezing. Many Gram-negative bacteria such as Escherichia coli, Salmonella spp. and Vibrio parahaemolyticus were affected by high salt concentrations. For Aerobacter aerogenes, sodium chloride solutions were the most lethal and the kill after freezing and thawing was independent of salt concentration over a wide range (Postgate and Hunter, 1961). Other salts such as lithium chloride, potassium chloride, sodium sulphate and potassium sulphate were equally harmful (Calcott and MacLeod, 1974). But phosphates were marginally protective (Postgate and Hunter, 1961; Ray and Speck, 1972).

The pH was another factor which profoundly influenced the survival during freezing. Georgala and Hurst (1963) studied the survival of Salmonella typhimurium and Demchick et al. (1982) of Staphylococcus aureus. Usually the acidic pH was more harmful to bacteria than alkaline pH.

The protective effect afforded by substances such as glycerol, sucrose etc. are well documented. (Postgate and Hunter, 1961; Calcott and MacLeod, 1974). Bacteria frozen in a protective environment showed greater recoveries. The protective action was thought to be osmotic (Hollander and Nell, 1954; Postgate and Hunter, 1961). Rey and Speck (1973) noticed that although cryoprotectants protected the cell against death, the number of damaged cells were high.

Straka and Stokes (1959) studied the various states of bacterial cell during exposure to low temperature. Though it was done in an arbitrary manner, this study brought out the existence of an intermediary state between life and death or unharmed and dead cells and were considered as "injured".

Cell injury due to cold shock or freezing has been observed by a number of workers for a variety of bacteria such as Escherichia coli (Straka and Stokes, 1959; Postgate and Hunter, 1963; Ray and Speck, 1972). Pseudomonas (Straka and Stokes, 1959; Postgate and Hunter, 1963; Arpai, 1962), Salmonella anatum (Ray et al., 1971), Aerobacter aerogenes (Postgate and Hunter, 1963) and Shigella sonnei (Nakamura and Dawson, 1962). Injury due to freezing occurred in Gram-positives also (Moss and Speck, 1963; Jackson, 1974). Most of the studies on the

injury of Staphylococcus spp., pertain to heat treatment which are not discussed here.

The various manifestations of the injury are impaired permiability, activation of certain enzymes, degradation of ribosome and increased sensitivity to certain selective agents. Reviews on these aspects have been compiled by Busta (1978) and Van Scothorst (1979).

2.3 Bacteriology of frozen fish and shellfish

Major areas that should be considered in the production of good quality frozen fishery products have been identified. They are quality of the raw material, handling/processing condition and post-process contamination.

2.3.1 Quality of the raw material

The quality of the raw material, to a great extent, determines the quality of the final product (Cann, 1977). The bacterial count increases considerably during the period after catch (Shewan, 1961; Lee and Pfeifer, 1977; Gillespie and Macrae, 1975), though most of the samples of freshly landed fish and prawn have the bacterial count within limit (Andrews et al., 1977; Zuberi and Quadri, 1980; Abeta, 1983; Lakshmanan et al., 1984). The method of catching (Watanabe, 1964; Novak, 1973),

seasonal variation (Raj and Liston, 1963; Iyer et al., 1970) etc. affect the final quality.

2.3.2. Effect of pre-process handling

Apart from the bacteria naturally associated with the fish and shrimp (non-specific types) considerable numbers get access to fish flesh as contaminants during various stages of processing. Some of them are of public health significance (Iyer et al., 1966). While primary contamination may occur up to the time of catching, secondary contamination begins with the onset of catching till it is finally processed.

Secondary contamination starts on the fishing vessel. Bacteria present on the deck, utensils, fish holds, ice and fish boxes get entry into the fish flesh and multiply therein (Watanabe, 1964; Castell, 1973).

Washing if carefully done can reduce the surface load of bacteria by 80-90% and with very careful hand washing up to 99% reduction is possible (Spencer, 1956; Shaikmahmud and Magar, 1956; Sreenivasan, 1959; Georgala, 1957a,b; Cann, 1977; Lee and Pfeifer, 1977; Ray et al., 1976). But washing with polluted inshore water may raise the bacterial load on fish (Watanabe, 1964). Also, washing generally did not have any effect

on generic distribution of microorganisms (Shewan, 1971a).

The use of chilled water has been advocated in washing process. Between chilled seawater at 0°C and tap water at 25°C, an increase in bacterial count was noted with tap water. But chilled water arrested bacterial multiplication, thereby arresting the bacteria (Pillai and Lekshmy, 1961). Other workers claimed that chilled water used for washing retarded bacterial growth, but the low temperature apparently favoured the selection of psychrotrophic spoilage bacteria (Shewan, 1971a, Lee and Pfeifer, 1977).

Ice is applied to reduce the temperature thereby delaying the spoilage of fish. But it acts as a dangerous vehicle in allowing microbial contamination. This possibility was studied by Georgala (1957a, b) and Iyer and Choudhuri (1966).

Compared to fish, more information is available on the handling of crustaceans. The beneficial effect of beheading the shrimp on the microbial quality has been reported widely. Green (1949a), Williams et al. (1952), Novak (1973) and Cann (1974) observed that for shrimp that landed alive beheading at sea was essential, to prevent autolytic changes. Contrary results are also reported (Koburger et al., 1973; Alvarez and Koburger,

1978). Qualitative studies on the microbial flora of shrimp stored as whole and headless showed that same bacterial groups predominated in approximately same percentage (Alvarez and Koburger, 1978).

There are numerous publications on the detailed microbiological evaluation of processing of shrimp (Harrison and Lee, 1969; Cann et al., 1971; Lee and Pfeifer, 1977; Zuberi et al., 1983) and other crustaceans, like crab (Philips and Peeler, 1972; Lee and Pfeifer, 1975; Ray et al., 1976). These studies indicated that microbial load increased after peeling and sorting operations and decreased after cooking, washing or brining. The Gram-positives were recovered with increasing frequency after each step (Harrison and Lee, 1969). The processing practices influenced the microbial load as well as the generic composition of the flora.

Farber and Lerke (1961) noted a positive correlation between diversity of the microbial flora in seafood and its freshness. This observed diversity indicated the lack of opportunity for a specific microorganism to dominate.

2.3.3 Bacteriology of freezing

2.3.3.1 Quantitative studies

The salient features of the microbiology of freezing

of fish was discussed by Tressler and Evers (1957). Freezing does not sterilize the fish; neverthless there is considerable reduction in bacterial population due to freezing.

Kiser and Beckwith (1942), studying the effect of fast freezing and storage at -28°C of mackerel, noticed that the muscle exhibited a decrease in count of 43.3% while the intestines showed a more striking reduction in bacterial count (97.9%). A reduction of 40-60% in bacterial population was noted by Pivnick (1949). Similar values were reported for tropical fishes also. (Sreenivasan, 1959; Bose, 1969; Jadhav and Magar, 1979; Cann, 1974; Zuberi et al., 1983).

While rate of freezing had no effect on bacterial death (Pivnick, 1949; Shewan, 1961), repeated freezing and thawing was found to be more lethal to bacteria.

The temperature of freezing had been the subject of some studies. But most of the workers used pure cultures of bacteria. It was found that lower the temperature of freezing, greater was the destruction (Hess, 1934; Kiser, 1944; Wieser and Osterud, 1945). At -195°C, there was no storage death at all. (Wieser and Osterud, 1945).

Kiser and Beckwith (1942) noticed that marked reduction in bacterial number occurred only after 48

hours. Pivnick (1949) reported bacterial reduction of 40-60% in 24 hours.

The method of packing, freezing and storage of headless shrimp was investigated by Fieger et al. (1956). The study indicated that freezing caused a greater reduction in bacteria in peeled shrimp than in unpeeled. Temperature fluctuations affected the quality. Samples stored at lower temperatures (-40°F) produced product of better quality than those stored at 0°C, while samples stored at +10°F showed definite signs of deterioration. Similar finding was reported for tropical sardine (Bose, 1969). In samples stored at -12.2°C (+10°F), the bacterial count after an initial fall continued to rise, whereas in fish stored at -23.3°C, the count decreased continuously.

Effect of immediate icing on bacterial numbers after freezing was studied for tropical prawns (Lekshmy et al., 1962; Bose, 1969). Backwater prawns which were uniced or iced for 1-2 days showed steady increase in count during frozen storage. But delay in icing for 3-4 days caused an increase in count after an initial decrease.

Effect of different glazes and packaging methods on the bacterial quality of the fishes from Bombay coast was studied (Jadhav and Magar, 1970). They found that both packaging method and glazes exerted some effect on bacteria. While ice water glaze increased the bacterial

number, others like citric acid, ascorbic acid and sodium chloride -glucose mixture tended to reduce the number of bacteria. Mathen et al. (1970) could not find any significant difference in bacterial count between glazed and unglazed shrimp. He suggested that for short term preservation (2 months), glazing was not necessary.

Phosphate treatment is practised in industry to prevent thaw drip. Mathen (1973) studied the effect of phosphate treatment on bacteria present in prawn and found no difference between treated and untreated prawn.

The difference between laboratory scale and commercial scale samples of frozen prawn with regard to the number of bacteria was studied (Novak, 1973). The percentage reduction of bacteria in laboratory frozen samples were fairly constant (89-99%), whereas in commercial samples, large variations were noted. The author attributed this to storage conditions and delay in freezing. Also, the laboratory frozen material possessed a lower count than commercial samples. For the factory frozen sample wide variation were noted among factories for the same product (Cann, 1977; Zuberi et al. 1983).

2.3.3.2 Qualitative changes of microflora on freezing

Earlier studies indicated that freezing imparted a selective action on the microbial flora of fish and various species were affected at different levels.

The Gram-positive bacteria were found to be more resistant to freezing and frozen storage. Kiser and Beckwith (1942) observed that Micrococcus and Achromobacter were frequently encountered in frozen mackerel. Pure culture studies showed that freezing and storage at -20°C for 20 days resulted in approximately 100% reduction of Achromobacter spp., while Micrococcus spp., withstood the temperature much better.

During one month's storage of Ocean perch at -15°C, Lee et al. (1967) noted the loss of all Gram-negatives. The Gram-positive species showed differing sensitivity to freeze damage. Among Gram-positives, Bacillus, Lactobacillus and Micrococcus species were most susceptible and coryneforms least affected.

Jadhav and Magar (1970) studying the bacterial flora of tropical fishes from Bombay coast found that spore-formers like Bacillus mesentericus group were very resistant to freezing as well as glazing by ascorbic acid, citric acid and sodium nitrite.

Microbiological characteristics of the frozen prawn imported from tropical countries were investigated by Kawabata et al. (1975). The study revealed that for shrimp also 70% of the flora constituted Gram-positives belonging to genera Micrococcus, Streptococcus, Staphylococcus, Microbacterium and Corynebacterium. The Gram-negatives

were few in number and belonged to the genera Flavobacterium/cytophaga, Pseudomonas, Moraxella and Acinetobacter. Zuberi et al. (1983) noted a predominance of Pseudomonas spp., in frozen shrimp followed by Micrococcus spp.

2.3.4 Effect of thawing

Frozen seafoods are accepted as highly stable and less prone to spoilage by bacteria. When food is defrosted, surviving bacteria are liberated and immediately begin multiplication resulting in chemical breakdown of the product. This problem has not been studied extensively and there is only limited information on this aspect.

Pivnick (1949) had done some work on the process of thawing of fish on the bacteriology of the material. He noticed that slowly frozen fish spoiled more quickly in the beginning (24 hours), while after storage for 3-6 months fast frozen fish spoiled more quickly. Although lag phase of the cultures immediately after freezing (24 hours) did not differ much from unfrozen control, the lag phase after 3-6 months of storage increased to 2-7 days. The author believed that the extended lag period was one of the reason for the extended shelflife of defrosted fish.

During thawing, bacteria start growing within the

temperature limit characteristic of the particular organism. The higher the external temperature, the more favourable it was for growth of most of the bacteria. Hence defrosting at low temperature ensured a low bacterial number (Winter and Wrinkle, 1949).

There are conflicting opinions on the spoilage rate of defrosted fish. Some believed that frozen material especially frozen vegetables and fruits spoiled more rapidly than fresh ones (Borgstrom, 1955). According to Shewan (1956), thawed fish spoiled at about the same rate as unfrozen control since freezing did not affect the biochemical properties of the surviving population. Similar observation was made by Sulzbacher (1952) for meat.

Compared to other food stuffs, there are greater chances of spoilage of seafood during and after thawing because the fish harbours a psychrophilic flora even from the beginning. Though freezing and immediate thawing killed as much as 50% of the psychrophilic microorganisms, sufficient numbers remained to promote spoilage of the frozen product. When frozen foods were defrosted and kept in the refrigerator, they underwent a spoilage similar to that of unfrozen fish (Liston, 1980).

Another factor to be considered in the process of thawing is the competition between different microorganisms.

Gunderson and his colleagues showed that at defrost, the growth of staphylococci was severely suppressed by saprophytic, psychrotrophic organisms (Peterson *et al.*, 1962a, b)

2.3.5 Effect of handling and processing on microorganisms of public health significance

Effect of various steps in handling and processing of seafoods with regard to bacteria of public health significance have been studied. Raj and Liston (1963) and Raj (1970) showed that raw material (seafood) entering the plant carried comparatively lower levels of bacteria of public health significance and each step introduced significant numbers of coliform, enterococci and coagulase positive staphylococci. The effect of processing, distribution and storage on the *Vibrio parahaemolyticus* count in oysters was studied by Thompson *et al.* (1976). Karuna Sagar *et al.* (1984) studied the shrimp undergoing processing for export to other countries for the presence of *Vibrio parahaemolyticus*. They noted that 51 out of 56 raw, 42 out of 50 processed and 54 out of 57 frozen samples contained *Vibrio parahaemolyticus* in quantities 10/g. No sample contained *Vibrio parahaemolyticus* greater than 10^2/g.

2.4 Bacterial spoilage of wet fish

Fish undergoes rapid spoilage when kept at ambient temperature for long time. It is only after bacterial attack that spoilage leading to unacceptable organoleptic changes occur. (Liston, 1964; Shewan and Murray, 1979). Endogenous changes that occur in fish postmortem are significant in conditioning the substrate for bacterial action to set in. The possibility of involvement of autolysis in spoilage mechanism is distant except in belly-burst phenomenon which occur in heavily feeding fishes or fish with high enzymic activity. In fatty fishes, rancidity is a major problem.

There are several reviews related to the bacteriology of fish spoilage. The classical work of Reay and Shewan (1949) summarizes some of the gross biochemical changes happening in some gadoid fishes. Other reports on this subject are those of Shewan (1962, 1977), Ingram (1971), Herbert et al. (1971), Liston (1980), Hobbs and Hodgkiss (1982) and Hobbs (1983).

It is exclusively proved that bacterial enzymes bringforth undesirable changes (Castell, 1973). According to him, fish could spoil from both inside (gut) and outside (skin and slime). The ability to spoil from inside depended on the abundance of food, partially

digested food and faecal matter, whereas spoilage from outside depended on the extent of bacterial contamination.

Recent finding is that as spoilage proceeds, there is a gradual invasion of flesh by bacteria from outer surface through peritonial lining. This was demonstrated by histological examination of fish flesh by Shewan and Murray (1979). They concluded that it was a slow process at chill temperature and the objectionable odours resulted mainly from bacterial activity in the surface slime and integuments, attacking low molecular weight components present on the skin and slime. On the otherhand, at higher storage temperature, actual penetration of the flesh occurred attacking muscle constituents.

Spoilage of tropical fish

Review of literature shows that some species of fish from tropics, when stored at chill temperature, showed extended shelflife of as much as six times that of north sea fish while some others spoiled as fast as north sea fish (Shewan, 1977). The presence of high content of lysozyme in the slime, pH of the postmortem muscle, difference in bacterial species existing on the skin surface etc. have been suggested as reasons for extended shelflife.

Simidu et al. (1969) and Gillespie and Macrae

(1975) studying sub-tropical and tropical fish found Pseudomonas to be the most important spoilage bacteria. Spoiled tropical shellfish from Gulf of Thailand and Mosambique carried Pseudomonas and Achromobacter groups (Cann, 1974). Other species of bacteria like Vibrio, Micrococcus, Alcaligenes, Flavobacterium/cytophaga were also reported to be active in spoilage in addition to Pseudomonas, Moraxella and Acinetobacter (Surendran, 1980). Chandrasekaran et al. (1985) found Vibrio spp., to constitute major flora of spoiled Indian prawn. The above studies established that spoilage flora found in warmer areas like Australia, Japan and India is similar to that existed in areas with low air/water temperature.

Surendran (1980) and Anand and Setty (1977, 1981) studied the psychrotrophic bacteria present in tropical fish and shellfish and the effect of chemical preservatives on these bacteria.

Significance of psychrophilic bacteria in spoilage of wet fish:

Psychrophilic/psychrotrophic bacteria are considered to be responsible for quality deterioration of chill-stored fresh fish. They constituted the normal flora of the skin, guts and gills of the marine fish. (Shewan and Murray, 1979). Control of spoilage organisms in fish is

mainly a deal with psychrophiles/psychrotrophs (Setty, 1985).

Castell et al. (1948) found that a definite relationship existed between bacterial count and keeping time when stored at 2°C and 3°C. They found that Pseudomonas, Achromobacter, Serratia and Proteus like organisms produced the most offensive spoilage at 3°C. The Flavobacteria, Micrococci and Myxobacteria produced off flavours more slowly.

Mossel and Ingram (1955) reported that food spoilage at low temperature below 10°C was primarily due to psychrophilic strains of Pseudomonas, Achromobacter and Flavobacterium species. This was further supported by other studies (Castell and Greenough, 1957; Castell et al., 1957; Shewan et al., 1960a; Miller et al., 1973). Sasajima and his co-workers studied the distribution of psychrotrophic bacteria in fresh fish and fishery products. They observed that most of the common genera were present among them. Distribution of psychrophilic bacteria was more in the fresh fish than fishery products (Sasajima, 1968).

The psychrophilic spoilage flora of the fish was also studied by Shaw and Shewan (1968). They reported that at 0-6°C, a high cell number of Pseudomonas spp., particularly groups II, III and IV prevailed in fish.

At 3°C, the Pseudomonas spp., constituted 25% of the bacterial flora.

Different kinds of spoilage resulting from growth of psychrotrophs were outlined by Splittstoesser (1976) who indicated that the type of food itself determined the spoilage pattern. Fish, as it harboured a psychrophilic flora even from the beginning, showed great tendency for spoilage. He also stated that only a few species of bacteria caused spoilage at low temperature, the major species being Pseudomonas. Out of a population of 10^6 - 10^7 bacteria/g, only 10-20% produced any noticeable change to food.

Some of the food handling/processing practices leading to the spoilage by psychrotrophs were reviewed by Gilliland et al. (1976) who pointed out that freezing and subsequent thawing during refrigerated storage resulted in slow growth of psychrotrophs ultimately leading to quality loss.

The role of Alteromonas putrefaciens in fish spoilage was emphasized by the work of Vanspreekens (1977). This organism, together with another facultative anaerobe Photobacterium spp., constituted more than 50% of the flora of marine fish at 3-4°C (Van Spreekens and Toepoel, 1981) and shellfish (Van Spreekens, 1977).

The type of end products responsible for putrid and offensive odours, substrates utilized by different bacterial species and rate of degradation among different spoilage groups have been studied by Chai et al. (1968), Herbert et al. (1975), Miller et al. (1973), Herbert and Shewan (1976) and Van Spreekens (1977). A list of psychrophilic bacteria associated with marine environment is given in Table 3. Table 4 gives a description of the spoilage pattern caused by important species of bacteria.

The Pseudomonas spp., have the shortest generation times at 0-5°C and possess an enhanced capacity to utilize various non protein nitrogen components of muscle fluids, resulting in rapid growth at these temperatures. The generation times of some of the members of these species are given in Table 5. They easily dominate other mesophilic types and cause spoilage in fish.

Chapter one of this thesis contains a brief introductory part regarding the status of freezing of fish and the scope of the present investigation.

An exhaustive survey of literature, regarding the past work carried out in the field of bacteriology of freezing of fish, is attempted in Chapter two.

In Chapter three, details of the relevant methods followed or techniques adopted have been dealt with.

Table 3

Psychrophilic bacteria associated with marine environs

Source	Temperature of growth (C)			Bacterial strain
	Minimum	Optimum	Maximum	
1. Silver salmon	-	15-20	20	Cytophaga psychrophila
2. Water	15	15	20	Vibrio marinus
3. Water Narrangan sett bay	-	-	-	Arthrobacter sp.
4. Water, North sea	-	-	< 20	Pseudomonas sp. Vibrio sp. and Spirillum sp.
5. Water, Antartic	-	7	13	Vibrio 18-300 and Vibrio 18-500
6. Water, Antartic	-	6-7	10	-
7. Water, Antartic	-	4	9	Vibrio sp. AP-2-24
8. Fish, Arabian sea	-	25-28	37	Achromobacter sp. Flavobacterium sp. Pseudomonas sp. Alcaligenes sp.
9. Fish, Arabian sea	-	0_15	20	Vibrio sp.
10. Haddock tissue	-	0-15	20	- (8.8%)

Nos. 1-7 taken from Baross and Morita (1978); No.8-9from Anand and Setty (1977)
No.10 from Makariose et al. (1984)

Table 4

*Spoilage potential of representatives of some important group of spoilers

Genus/species	Odours		TMA	HX
	Fish	Shrimp		
Pseudomonas Groups I/II	Boiled milk, rubbery musty vegetables, cabbage, onions, pungent aromatic cheesy, fishy, sour, sweet, fruity, rotten onions, dirty rabbit hutch, rotten cabbage, cesspit (19)^{H}	musty, musty hay musty cellar, fruity, aromatic, NH_3, pungent cabbage, rotten cabbage (19)	- (28)	- (28)
Alteromonas putrefaciens	Cabbage-like, rubbery, aromatic, sour, fishy, dirty rabbit hutch, rotten cabbage, cesspit, mushrooms (32)	musty, musty cellar (a little) NH_3, cabbage-like, dirty rabbit hutch, rotten cabbage (32)	+ (62/63)	+ (62)
Alteromonas sp. "typical shrimp spoilers"	Neutral, aromatic, fresh fish, marinated onions, a little cabbage-like, spoiling vegetables (23)	musty, cabbabe-like, NH_3, rotten cabbage, H_2S, dirty rabbit hutch (23)	- (28)	+ (21)
Moraxella	Fresh fish, a little aromatic, fruity, sour, boiled milk, fishy, hay, rubbery, musty, acceptable for longer periods (18)	A little aromatic, a little musty, cheesy, neutral, fruity, sometimes NH_3, rotten cabbage (18)	+ (75/234) 32%	- (87)
Photobacterium	Fresh fish, buttermilk a little sour, sometimes rubbery, sometimes a little musty (39)	fresh shrimp, sometimes fruity, acceptable for longer periods (15)	+ (66/67)	+ (64/65)

*After Van Spreekens and Toepoel (1981); H- Numbers of isolates tested given in brackets

Table 5

Reported generation times for some psychrophilic bacteria

Bacterial strain	Source	Generation time (h) at				
		0°C	2-2.5°C	4-5°C	10°C	20°C
Clostridium hastiforme	Milk	-	-	73	25.5	-
Clostridium sp. 61	River mud	17	-	-	9.0	3.5
Bacillus W 25	Soil mud	23	-	8.5	6.0	2.5
Pseudomonas 92	Dairy product	26.6	-	11.7	5.4	1.7
Pseudomonas fluorescens	Fish	30.2	-	6.7	-	1.4
Pseudomonas fragi	Dairy product	11.3	7.7	5.0	2.6	1.1
Pseudomonas sp. (1-3 b)	Chicken	10.3	8.0	7.0[a]	2.7[b]	1.6
Gram-negative rod	Fish	20.0	-	7.6[a]	1.9[b]	-
Pseudomonas sp. (451)	Meat	-	13.8	9.7	4.0	1.2
Pseudomonas fluorescens		30.20	-	-	-	-
Pseudomonas fluorescens		26.4	-	10.65	-	-
Pseudomonas sp.		10.33	-	-	2.66	-
Pseudomonas fluorescens		-	-		4.2 to 8.20	2.66

After Shewan and Murray (1979); a. Generation time determined at 6°C
b. Generation time determined at 12°C.

Presentation of the data, discussions of various aspects and inferences are contained in Chapter four. The following aspects have been investigated in detail.

i. Factors affecting the recovery of bacteria from fresh/frozen fish and shellfish.
ii. Bacteriology of newly caught fish.
iii. In-vitro studies on the effect of freezing on bacterial isolates from fish.
iv. Bacteriology of freezing of fish/shellfish.

Some of the readily available fishes like oil sardine (Sardinella longiceps), mullet (Mugil cephalus), Indian mackerel (Rastrelliger kanagurta) jew fish (Johnius dussumeri) and prawn (Metapenaeus dobsoni) were used for evaluating the methodology. But samples used in the later part of the work were restricted to two types namely, Indian mackerel (Rastrelliger kanagurta) and prawn (Metapenaeus dobsoni).

A brief summary of the results and discussion is given in Chapter five and various references cited in Chapter six.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Media

The bacteriological media used in the study were of two categories.

3.1.1.1 Dehydrated media

Dehydrated media from Oxoid, England and Difco Laboratories, U.S.A. were used. The following media were used.

Trypticase Soy Agar (TSA)

Baird-Parker Agar (BP)

Desoxycholate Lactose Agar (DLA)

Eosine Methylene Blue Agar (EMB)

Brain Heart Infusion Agar (BHIA)

Brain Heart Infusion Broth (BHIB)

Thiosulphate Citrate Bile Salt Sucrose Agar (TCBS)

EC Medium (EC)

3.1.1.2 Media compounded in the laboratory

Sea water Agar (SWA)

Peptone, 10 g; ferric phosphate, 50 mg; agar agar powder, 15 g; sea water (aged, filtered), 1 litre; pH 7.2. Sterilized at 1.05 kg/cm^2 gauge pressure for 15 min.

Sea water peptone (SWP)

Peptone, 10 g; potassium nitrate, 200 mg; Sea water (aged and filtered), 1 litre; pH 7.2. Sterilized at 1.05 kg/cm^2 gauge pressure for 15 min.

Cysteine-peptone medium

Peptone, 10 g; Cysteine Hcl, 0.1 g; sodium sulphate, 0.5 g; sea water (aged, filtered), 1 litre; pH 7.2. Sterilized at 1.05 kg/cm^2 gauge pressure for 15 min.

Tryptone Broth (TB)

Tryptone, 10 g; beef extract, 3 g; sodium chloride, 5 g; distilled water, 1 litre; pH 7.2. Sterilized at 1.05 kg/cm^2 gauge pressure for 15 min.

Tryptone glucose Agar (TGA)

Tryptone, 5 g; beef extract, 3 g; dextrose, 1 g; sodium chloride, 5 g; agar agar powder, 15 g; distilled water, 1 litre; pH 7.2. Sterilized at 1.05 kg/cm^2 gauge pressure for 15 min.

Antibiotic Agar (ABA)

Peptone, 5 g; yeast extract, 5 g; beef extract, 5 g; sodium chloride, 10 g; agar-agar powder, 15 g; distilled water, 1 litre;

pH 7.2. Sterilized at 1.05 kg/cm^2 gauge pressure for 15 min.

Peptone Sucrose Agar (PSA)

Peptone, 10 g; sucrose, 10 g; sodium chloride, 10 g; bromothymol blue, 0.025 g; agar-agar powder, 15 g; distilled water, 1 litre; pH 7.2. Sterilized at 1.05 kg/cm^2 gauge pressure for 15 min.

Minimal Agar (MA)

Stock solution I

Dipotassium hydrogen phosphate, (K_2HPO_4), 14 g; Potassium dihydrogen phosphate, (KH_2PO_4), 6 g; Sodium citrate ($Na_3C_6H_5O_7$ $2H_2O$), 200 mg; magnesium sulphate ($MgSO_4$ $7H_2O$), 200 mg; ammonium sulphate ($(NH_4)_2$ SO_4), 2 g; distilled water, 80 ml; pH 7.2. Sterilized at 1.05 kg/cm^2 gauge pressure for 15 min.

Stock solution II

Glucose, 10 g; distilled water, 50 ml. Sterilized at 1.05 kg/cm^2 gauge pressure for 15 min.

Agar base

Agar-agar powder, 1.5 g; distilled water, 95 ml. Sterilized at 1.05 kg/cm^2 gauge pressure for 15 min.

When required 4 ml of stock solution I and one ml of stock solution II were added to 95 ml of melted agar (cooled to 45°C) and mixed well before pouring into petridishes.

Skim Milk Agar

A sterile solution of skim milk powder in distilled water (sterilized at 0.07 kg/cm^2 for 12 min) was added aseptically to nutrient agar base, melted and cooled to 50°C. The final concentration of the skim milk in nutrient agar was adjsted to 10%.

Other media used in course of the study such as nutrient agar, nutrient broth, K F agar, Violet Red Bile Agar, diluents etc. were prepared according to the method outlined in FDA Analytical Manual (FDA, 1978) and ICMSF (1978).

3.1.2 Chemicals

Ingredients for the bacteriological media such as peptone, yeast extract, beef extract, agar powder etc. were from Oxoid (England) or Difco (USA).

Other chemicals used were of BDH (England), SISCO (India) or Merck (Germany) brands and were of analytical reagent grade.

3.1.3 Fish and Prawn

The fishes used in the first part of the study (4.1) were oil sardine (Sardinella longiceps), mackerel (Rastrelliger kanagurta), mullet (Mugil cephalus) and jew fish (Johnius dussumeri). Two types of prawn namely, Metapenaeus dobsoni (M. dobsoni) and Parapeneopsis stylifera (P. stylifera) were also used. Subsequent studies (4.2, 4.4 and 4.5) were carried out with only mackerel (R. kanagurta) and M. dobsoni. The fishes/prawns were procured from the department vessel operating off Cochin. The fish for bacteriological analysis was transferred as aseptically as possible into wide mouthed bottles, which were then securely closed and kept under ice in boxes during transportation. These samples were brought to the laboratory within 2-4 hours after catch and analysed immediately.

The fish/prawn for freezing studies were subjected to various treatments the details of which are given elsewhere in the thesis.

3.1.4 Bacterial strains

The bacterial cultures included in the study were,
i. Bacteria isolated from fish/prawn or its products and maintained by the culture collection of Central Institute of Fisheries Technology. Both marine bacteria and pathogenic/indicator bacteria were available.

ii. Bacteria isolated and identified in course of this work. These bacteria were isolated from mackerel (Rastrelliger kanagurta) and prawn (Metapenaeus dobsoni). They were maintained on SWA slants.

iii. Bacteria received from National Collection of Marine Bacteria (NCMB) of Torry Research Station, Aberdeen. They were used as reference strains.

A list of various bacteria used in this study is given in Tables 6 to 8.

3.2 Methods

3.2.1 Standardization of method for the recovery of bacteria from fish/prawn

Fresh/frozen fish and prawn were used in this study. Fresh fish/prawn was collected as per methods outlined in 3.1.3. Frozen blocks were prepared in the laboratory (3.2.7).

3.2.1.1 Plating methods

Two methods viz. conventional pour plate method and spread plate method were compared.

Muscle homogenates of the raw/frozen material were prepared by grinding about 10 g of the skin and muscle with 100 ml of n. saline in a sterile mortar. This formed 10^{-2} dilution. Subsequent dilutions were

Table 6

Bacterial type cultures from NCMB, Aberdeen

Sl.No.	Culture Number	Bacterial strain
1	NCMB-129	*Pseudomonas fluorescens*
2	NCMB-320	*Pseudomonas fragi*
3	NCMB-19	*Alteromonas haloplanktis*
4	NCMB-398	*Pseudomonas* sp.
5	NCMB-132	*Moraxella* like strain
6	NCMB-308	*Moraxella* like strain
7	NCMB-244	*Flavobacterium* sp.
8	NCMB-251	*Cytophaga/ Flexibacter* group
9	NCMB-407	*Vibrio anguillarum*
10	NCMB-1281	*Vibrio fischeri* (luminous)
11	NCMB-13	*Micrococcus* sp.
12	NCMB-1491	*Planococcus* sp.

Table 7

List of Pathogenic/indicator bacteria

Sl. No.	Culture No.	Bacterial strain	Source
1	E-1	*Escherichia coli*	CIFT culture collection
2	E-2	*Escherichia coli*	"
3	Sa-21	*Salmonella anatum*	"
4	ST-3	*Salmonella typhimurium*	"
5	S-7	*Staphylococcus aureus*	"
6	S-8	*Staphylococcus aureus*	"
7	Vp-9	*Vibrio parahaemolyticus*	"
8	Vp-11	*Vibrio parahaemolyticus*	"
9	Sf-2	*Streptococcus fecalis*	"
10	Sf-4	*Streptococcus* sp.	"
11	Sc-15	*Salmonella* sp.	"
12	CM-13	*Salmonella* sp.	"
13	PN-38	*Escherichia coli*	Prawn
14	SP-111	*Escherichia coli*	"
15	PD-12	*Staphylococcus* sp.	"
16	PW-15	*Staphylococcus* sp.	"
17	SC-9	*Streptococcus* sp.	Mackerel
18	SPM-1	*Streptococcus* sp.	Prawn

Table 8

List of bacterial strains isolated from mackerel/prawn

Sl. No.	Culture No.	Source	Bacterial strain
1	2PW-5	Frozen mackerel	Pseudomonas sp. (Psychrotrophic)
2	SM-4	Skin of fresh mackerel	Pseudomonas sp. (Psychrotrophic)
3	3LW-5	Frozen prawn	Pseudomonas sp. (Psychrotrophic)
4	LS-1	Frozen prawn	Pseudomonas sp.
5	SS-4	Surface of mackerel	Pseudomonas sp.
6	S.G-8	Gills of mackerel	Pseudomonas sp.
7	PW-4	Mackerel	Vibrio sp.
8	RD2T	Fresh prawn (P.D.)	Vibrio sp.
9	PD-6	Fresh prawn (P.D.)	Vibrio sp.
10	2LW-6	Fresh prawn	Vibrio sp.
11	PD4T	Mackerel	Vibrio sp.
12	LW-6	Fresh prawn	Moraxella sp.
13	2PW -4	Frozen mackerel	Moraxella sp. (Psychrotrophic)
14	PW-9	Fresh mackerel	Moraxella sp. (Psychrotrophic)
15	SM-21	Frozen prawn	Moraxella sp. (Psychrotrophic)
16	LM-3	Frozen prawn	Moraxella sp. (Psychrotrophic)

Table contd.

Table 8 contd.

Sl. No.	Culture No.	Source	Bacterial strain
17	LW-2	Fresh prawn	*Acinetobacter* sp.
18	MS-8	Fresh mackerel	"
19	2PW-7	Frozen mackerel	"
20	PD-8	Frozen prawn	"
21	SM-11	Frozen prawn	"
22	PW-24	Frozen prawn	"
23	CP-4	Frozen mackerel	*Micrococcus* sp.
24	SM-14	Fresh mackerel	"
25	PD-13	Prawn	"
26	2LWP-2	Frozen prawn	*Micrococcus* sp. (Psychrotrophic)
27	CP-2	Fresh prawn	*Micrococcus* sp.
28	3PW-1	Frozen mackerel	"
29	SM-10	Fresh mackerel	*Bacillus* sp.
30	LW-15	Gut of mackerel	"
31	PW-21	Peeled and deveined prawn	"
32	8MG-4	"	"
33	6MX-7	Frozen mackerel	"
34	IMS-7	Skin of mackerel	*Flavobacterium* sp.
35	IMS-8	"	"
36	2MS-18	Frozen mackerel	"
37	4-MS-11	"	"
38	4-MG-8	Gut of frozen mackerel	"
39	7NG-4	"	"

prepared in the same diluent by mixing 1 ml of the sample dilution with 9 ml of diluent in a vortex mixer (Remi, India). Sea water agar (SWA) was used for sampling fresh fish/prawn and tryptone glucose agar (TGA) for frozen fish/prawn.

The agar media for spreading had been poured 2 hours before the experiment and excess moisture was evaporated by keeping the plates at 45°C for one hour. Care was taken to prevent syneresis fluid to accumulate on the surface of the agar as it may lead to spreading of the colonies. 0.5 ml of the appropriate dilution was spread evenly on the agar surface with alcohol-flamed bent glass rod.

The spread plates were equilibrated with the temperature of incubation to be used in the experiment, prior to inoculation.

Pour plating was done using 1 ml of the inoculum. Agar media, previously molten and maintained in molten condition by keeping in a thermostatic water bath (Tempo, India) at 45°C, was used for pouring the plates. All samples were poured in triplicate.

3.2.1.2 Incubation temperature

One set of plates from each method was kept at

29±2°C (RT) and another set at 37°C in the incubator. In addition a set of plates were kept at 5°C for psychrophilic count.

3.2.1.3 Incubation period

The plates were incubated at 29±2°C (RT) for 48 hours, 37°C for 48 hours and 5°C for 21 days.

To study the effect of incubation period on bacterial count, plates at 29±2°C were kept at that temperature for 4 days and counts were taken after 24 hours, 48 hours, 72 hours and 96 hours.

3.2.1.4 Diluents

This part was carried out as a separate experiment. The diluents included in the study were

1. Distilled water
2. Sea water (full strength)
3. Normal saline (0.85% w/v Nacl)
4. Phosphate buffer (Medium No.90, ICMSF, 1978)
5. Peptone water (0.1% w/v of the peptone in distilled water)
6. Ringer's solution, Quarter strength (Medium No.97, ICMSF, 1978)

Additionally the following three combinations of these diluents were also tried.

1. Peptone-saline or PS (Media No.83, ICMSF, 1978)
2. Peptone-phosphate buffer or PPB (0.1% peptone in phosphate buffer)
3. Phosphate buffered saline or PBS (Medium No.91, ICMSF, 1978)

Minor variations were made in the preparation of the inoculum to maintain the uniformity of the sampling material. 50 g of the muscle was dry-grinded into a paste in a sterile mortar. 10 g lot of this material was transferred to separate sterile mortars and homogenized with the different diluents under study. Further dilutions were prepared in 9 ml aliquots of the respective diluents. The serial dilutions were pour plated in SWA/TGA and incubated for 48 hours. SWA was used for fresh fish and TGA for frozen fish. The number of bacteria per g was calculated using the relation.

$$\text{total bacterial count} = \frac{\text{Average count x dilution}}{\text{weight of sample}}.$$

For counting the plates, the principles, described in FDA (1978) was followed throughout the study.

3.2.1.5 Determination of hold up time of diluents

3.2.1.5.1 Fresh/frozen fish and prawn

The diluents used in this study were distilled water, sea water, phosphate buffer and n.saline. Details

regarding the preparation of inoculum, media, incubation etc. were as in 3.2.1.4. The zero hour count represented the bacterial count taken immediately after the preparation of the serial dilutions. In all cases, time required for the preparation of the dilutions did not exceed 4 min. The dilutions were then kept at room temperature with frequent shaking and bacterial counts determined periodically at the end of 10, 20, 30 and 40 minutes.

3.2.1.5.2 Pure culture studies

Pure cultures of bacteria listed in Table 7 and 8 were used. Cell suspensions of the individual cultures were prepared as given in 3.2.6.1. 1 ml quantities were used to inoculate the various diluents. The rest of the procedure was the same as that adopted for fresh/frozen fish and prawn (3.2.1.5.1).

3.2.1.6 Growth temperature studies

The experiment was carried out with cultures isolated from SWA/TGA plates and incubated at 5°C, RT (29±2°C) and 37°C. 0.5 ml of a uniform cell suspension of the cultures in distilled water (3.2.6.1) was inoculated into duplicate tubes of SWP/NB as the case may be. These tubes were incubated at 0°C, 10°C, 20°C, 30°C, 37°C, 45°C

and 56°C and observed daily for the appearance of growth as indicated by turbidity. Results were recorded.

3.2.2 Enumeration of bacteria in fresh fish/prawn

3.2.2.1 Enumeration of total aerobic bacteria in fresh fish

The total aerobic bacteria present on the skin with muscle, gills and intestine of mackerel was estimated.

3.2.2.1.1 Skin with muscle

4-5 fishes were randomly selected and about 10 g of skin with muscle was cut aseptically from just below the dorsal fin on the dorsal side.

3.2.2.1.2 Gills

About 3 to 5 g of the gill tissue from fresh mackerel (6-8 nos.) were taken.

3.2.2.1.3 Intestine

The intestine with contents - the part between stomach and anus was used (about 1 g).

The material was homogenized well with 100 ml of n.saline and serially and decimally diluted to appropriate dilutions. 0.5 ml of the inoculum was spread-plated on the surface of SWA and TGA plates in triplicate. Plates were incubated at RT(29±2°C) for 2 days and counted. This count was taken as the total

aerobic count of the sample. An additional set of plates, incubated at 37°C for 2 days, represented the mesophilic count and the count at 5°C (21 days) was taken as the psychrotrophic count.

Counting the colonies and calculating the total bacterial count was as in 3.2.1.4.

3.2.2.2 The total aerobic bacteria in prawn

The total aerobic bacteria on flesh and intestines was determined.

3.2.2.2.1 Body meat

After cutting of head portion and removing the shell, the flesh alone was taken under aseptic conditions with scissors and forceps.

3.2.2.2.2. Intestine

Intestine with contents of about 15-25 numbers was collected aseptically so as to make about 1 g of the sample.

The details of the procedure for bacterial enumeration were as given for mackerel (3.2.2.1).

3.2.2.3 Pathogenic bacteria in fish/prawn

The inoculum was the same as used for determination of total aerobic count.

3.2.2.3.1 Total coliforms and *E. coli*

The procedure given in FDA was followed (FDA, 1978)

1 ml aliquots of the appropriate dilutions were transferred to lauryl sulphate tryptose broth (10 ml) and incubated for 48±2 h at 37°C. Gas positive tubes were subjected to confirmatory test by transferring a loopful to brilliant green lactose bile broth (BGLB) tubes and incubating for 48 h at 37°C and most probable number (MPN) was calculated from the positive tubes. A loopful from gassing LST tube was transferred to Escherichia coli (EC) broth and incubated for 48 h at 45.5°C and MPN of *E. coli* was calculated on the basis of gas positive EC tubes. They were further confirmed by Gram stain and biochemical (IMVIC) tests.

3.2.2.3.2 *Staphylococcus aureus*

The number of *Staphylococcus aureus*/g was determined by the direct plating method using Baird-Parker medium. (FDA, 1978). 0.5 ml of the inoculum was surface plated and incubated for 48 h at 37°C. Typical colonies of coagulase positive *S. aureus* were counted. Representative colonies were subjected to coagulase test. The number of coagulase positive colonies per ml was calculated and multiplication by dilution factor gave the count in the original sample.

From this the number of coagulase positive staphylococcus aureus per g was determined.

3.2.2.3.3 Faecal streptococci

The medium used was K.F, streptococcus agar. 1 ml of the inoculum was pour plated on K.F. agar and plates were incubated at 37°C for 48 h. Typical colonies were counted and confirmation of the culture was performed by the procedure of ICMSF (1978).

3.2.2.3.4 Salmonella

The sample was screened for the presence of Salmonella by the method outlined in FDA (1978).

3.2.2.3.5 Vibrio parahaemolyticus

The sample was screened for the presence of Vibrio parahaemolyticus by the procedure outlined in FDA (1978). 50 g of the fish tissue or 50 g of the entire meat with intestinal content of shrimp was used. This was homogenized with 450 ml of 30% Nacl solution and decimal dilutions were transferred to glucose salt teepol broth (GSTB). (Single strength glucose). At end of 8-18 h, a loopful from gas positive tubes were streaked on thiosulphate citrate bile salt sucroseagar, and incubated for 18 h at 37°C. The typical colonies, if any, were

subjected to Gram stain and further tests for confirmation.

3.2.3 Enumeration of bacteria in frozen fish/prawn

3.2.3.1 Total aerobic bacteria

In the case of mackerel, the skin and muscle portion and intestinal contents were subjected to analysis.

After scraping off the adhering ice crystals with sterile scalpel, about 10 g of the skin with muscle of the frozen mackerel was scooped out aseptically, using sterile knife and forceps. The muscle was homogenized with 90 ml of n.saline and serial dilutions were prepared in the same diluent. 0.5 ml portions of appropriate dilutions were spread-plated on the surface of SWA and TGA. Plates were incubated at RT (29±2°C) and counts were taken at the end of 48 h and total aerobic count recorded. A set of plates were also kept at 37°C and 5°C and incubated for 2 and 21 days respectively and counts taken. The plates, incubated at 5°C, were then reincubated at RT (29±2°C) for one day and this represented the 5/30 count. The bacteria in the intestine (about 1 g) was determined by the procedure described as above.

The total aerobic bacteria of the frozen prawn (meat with intestine) was determined by the same method as that of fish.

3.2.3.2 Enumeration of pathogenic/indicator bacteria

The method followed was the same as that of fresh fish/prawn and is given in 3.2.2.3.

3.2.4 Enumeration of bacteria in the water samples

The water used in the various processing steps was subjected to the bacteriological analysis for total aerobic count and pathogenic/indicator bacterial count. The method adopted was that of APHA (1970).

3.2.5 Isolation of the bacterial cultures

About 30 to 50 cultures were picked from sea water agar/tryptone glucose agar plates as the case may be, taking care to include colonies of differing morphology in their relative proportion. Colonies growing on SWA plates were transferred to sea water peptone (SWP) and those from tryptone glucose agar plates to tryptone glucose broth (TGB). These were incubated at the corresponding incubation temperature used for initial isolation i.e. the colonies isolated from the plates at RT were incubated at RT. The period of incubation were 2 days for cultures kept at RT and 37°C and 14 days for those kept at 5°C. Those cultures, showing visible growth after the completion of incubation period, were taken on to SWA or NA slants as the case may be.

The cultures were purified by repeated streaking on the respective agar media. Finally the pure cultures were maintained on SWA/NA slants at 5°C.

3.2.5.1 Morphological studies

For Gram-staining, smears were made from 18-20 h old cultures, grown on SWA/TGA slants at RT (29±2°C)/ 37°C. In the case of cultures grown at low temperature (the cultures were observed daily for visible growth), slants showing visible growth were used. In most cases, growth could be observed after 7 days on incubation at 5°C. The method used for Gram staining was the Huckers modification of Grams method (Anon, 1957). Along with Gram reaction, motility was observed by hanging drop method (Anon, 1957). The arrangement of flagella was studied by Leifson's method (Leifson, 1960). Presence of spore was observed by the Gram staining of 48-72 h old cultures and further confirmed by the method of Dorner with Snyder's modification (Anon, 1981) using carbol fuchsin and nigrosin.

3.2.5.2 Biochemical characteristics

The presence of cytochrome oxidase was tested by the method of Kovacs (1956). The oxidative/fermentative attack on glucose was done by the method of Hugh and

Leifson (1953). Indole production was detected in tryptone broth using Kovac's reagent (ICMSF, 1978). Amylase activity was detected by the procedure of Iyer and Karthiayani (1964). Nitrate broth was used to study nitrate reduction (FDA, 1978). To study the production of hydrogen sulphide, lead acetate paper strips were hung over cysteine peptone medium. Methyl red, voges-Proskauer and utilization of citrate were done by the method of FDA (1978). Phosphatase activity was studied by the method of Vanspreekens (1977), and nuclease activity by the method given in FDA (1978).

3.2.5.3 Identification of the bacterial culture

The cultures, isolated during the studies, were identified only up to the generic level. For identification, the schemes proposed by Shewan et al. (1960b), Lee and Pfeifer (1975) and Surendran (1980) were consulted in addition to Bergey's manual (Buchanan and Gibbons, 1974). The identity was confirmed by comparison with reference strains (Table 6).

3.2.6 In-Vitro studies on freezing, frozen storage and thawing with isolated cultures

Cultures studied were those isolated in course of this investigation. 4-6 representative cultures were used from each genera. In addition, for pathogenic/

indicator bacteria, cultures obtained from culture collection of CIFT were also used (Tables 7 and 8)

3.2.6.1 Preparation of inoculum

The following procedure was adopted for preparation of inoculum. Erlenmeyer flaks (150 ml capacity), containing 100 ml of sea water peptone/tryptone glucose broth, were inoculated with a loopful of marine/pathogenic cultures from 18 h old SWA/NA slants, maintained at 29±2°C/37°C. Cultures in exponential phase of growth (absorbance of 0.45-0.5 at 600nm) were subcultured into fresh flask of respective medium. Three such subcultures were made before the culture was used as inoculum. The final culture was allowed to grow up to stationary phase (18 h). The culture suspensions were centrifuged at 4000 r.p.m. for 15 min, washed three times with equal quantity of sterile distilled water and finally suspended in sterile distilled water so as to get a final cell density of 10^7 - 10^8 cells/ml (0.2 - 0.3 absorbance at 600 nm). 1 ml of this suspension was used to inoculate various substrates.

3.2.6.2 Freezing

The inoculated material was frozen by keeping at -39±2°C or -20±2°C. The freezing rate was about 20°/h or 1°/3 min. After freezing they were stored at -20±2°C.

3.2.6.3 Enumeration of frozen and thawed cells

Frozen suspensions were retrieved at definite intervals. They were thawed (6-8°/min) in running water and spread plated using 0.1 ml quantities of decimal dilutions. Triptic soy agar was used for pathogenic/ indicator isolates and sea water based TSA for marine bacteria and plates were incubated for 2 and 21 days at RT/37 and 5°C respectively. The cell density per ml was estimated by multiplying colony count with dilution factor. During storage studies purity of the culture was confirmed by randomly isolating the colonies from the plates and studying their identity.

3.2.6.4 Preparation of fish muscle homogenate (Fish muscle medium - FMM)

The method of Matches et al. (1971) was used for the preparation of fish muscle homogenate (FMM). The material was distributed in 10 ml quantities in test tubes sterilized at 0.7 kg/cm^2 for 20 min and stored at 8°C till used.

3.2.6.5 Determination of survival rate at different temperatures

Pure strains of bacteria, listed in Tables 7 and 8, were used. A standardized cell suspension (of known cell density) was inoculated into tubes of fish muscle

medium and nutrient broth/sea water peptone broth (NB/SWA). The marine isolates were inoculated into SWP broth and pathogenic/indicator bacteria into NB. Tubes were kept at -39±2°C, -20±2°C and +7°C. These were withdrawn at definite intervals and surviving cell estimated as described in 3.2.6.3.

3.2.6.6 Determination of survival in different suspending fluids

The suspending fluids were distilled water, sea water (full strength) n.saline, phosphate buffer, peptone water (1% w/v of aqueous solution) and fish muscle medium. The tubes were frozen at -39±2°C and stored at -20±2°C. Rest of the procedure was as in 3.2.6.5.

3.2.6.7 Determination of the survival of mixed population

3.2.6.7.1 Pseudomonas and Moraxella species

The above combinations were studied and were carried out with a single strain of individual bacteria. The behaviour of these two strains towards the antibiotic penicillin was used to distinguish between Pseudomonas and Moraxella species. The Pseudomonas sp. was not sensitive to 3 µg of penicillin, whereas Moraxella was very much sensitive (Lee and Pfeifer, 1975).
The sensitivity was further confirmed by

repeated streaking on antibiotic agar with 3 µg of penicillin per ml of AB agar.

The procedure was standardized by repreated trials with fresh(unfrozen)and frozen cultures of the two bacterial species. Individual cultures and mixture of the two organisms (with known cell number) were plated on antibiotic agar (AB) and antibiotic agar having 3 ug/ml of added penicillin (ABP). The numbers of colonies developing (after 2 days at RT) on AB consisted of both sensitive and insensitive strains,while the colonies developing on ABP consisted of insensitive strain only. Hence, the difference between AB and ABP gave the count of sensitive strain. Survival of each type was calculated as

$$\% \text{ survival of sensitive strain (}\underline{\text{Moraxella}}\text{ sp.)} = \frac{\text{AB count - ABP Count x 100}}{x}$$

$$\% \text{ survival of resistant strain (}\underline{\text{Pseudomonas}}\text{ sp.)} = \frac{\text{ABP count x 100}}{y}$$

where x and y are the initial numbers of the sensitive and resistant species in the mixture.

After the preliminary trials, tubes of fish muscle medium were inoculated with known quantities of the two strains . It was frozen at -39±2°C and tubes were stored at -20±2°C. Periodically, tubes were withdrawn and the percentage of survivors of the two strains

calculated by the method described above.

3.2.6.7.2 Pseudomonas sp. and non-pigmenting Micrococcus sp.

The Pseudomonas sp. readily fermented sucrose and the Micrococcus sp. failed to do so. This reaction was used to differentiate the two strains. A medium peptone sucrose Agar (PSA) containing bromothymol blue indicator was used. The colonies of Pseudomonas turned yellow, whereas that of Micrococcus sp. were colourless. The percentage of each was calculated. The method of inoculation, freezing menstrum etc. were as in 3.2.6.7.1. The validity of the experiment was confirmed by repeating the experiments with the two cultures of known cell density.

3.2.6.7.3 Pigmented and non-pigmented strains.

Yellow pigmented and non-pigmented Micrococcus spp. were used. The medium used was skim milk agar. The number of pigmented and non-pigmented colonies, developing after 3 days at 29±2°C (RT), could be distinguished visually. They were counted separately and the percentage of each calculated. The details of freezing method, menstrum etc. was as in 3.2.6.7.1

3.2.6.8 Studies on the factors influencing the survival of bacteria during freezing and frozen storage

This experiment was performed with a limited number

of strains only viz. Pseudomonas spp. Vibrio spp. Escherichia coli and Micrococcus spp. Three species of each strain were studied.

3.2.6.8.1 Effect of initial cell number

The experiment was carried out in fish muscle medium. The inoculum prepared as in 3.2.6.1 was adjusted to various cell concentrations ranging from 10^6 to 10^9 cells/ml and was used to seed the substrate. One set of tubes were frozen and stored at -39±2°C and another set at -20±2°C. Tubes were withdrawn immediately after freezing (6 h) and at the end of one month. Surviving cells were estimated as given 3.2.6.2.

3.2.6.8.2 Effect of hydrogen ion concentration (pH)

Since fish muscle is prone to frequent changes in pH during storage period, this part of the studies were carried out in beef extract solution (aqueous, 1%). The cell suspensions of known cell density (3.2.6.1) of the test strain were introduced into ten ml aliquots of beef extract, adjusted previously to pH levels ranging from 3 to 9 using a pH meter. Rest of the procedure regarding freezing, enumeration etc. were as in 3.2.6.8.1.

3.2.6.8.3 Effect of age of cells

The strains Pseudomonas, Vibrio and Micrococcus were grown in SWP and E. coli in nutrient broth. SWP tubes were incubated at RT and NB at 37°C. Cells were harvested at definite intervals between 2-24 h so as to get cells of different age (phase of growth). A cell suspension of 10^4 - 10^5 cells were used for inoculation. Other details regarding freezing, substrate etc. were as in 3.2.6.8.1.

3.2.6.8.4 Effect of added chemicals

Chemicals studied were sucrose, glycerol, sodium chloride and potassium chloride. Sterile aqueous solutions of these substances at different concentrations, ranging from 2.5 mg to 25 mg/ml, were prepared. Cell suspensions of known cell density were introduced into tubes of 10 ml aliquots of the preparations. Rest of the procedure such as freezing, enumeration etc. were as in 3.2.6.8.1.

3.2.6.9 Determination of the cell injury during freezing and frozen storage

The study was carried out with selected strains of Pseudomonas spp., Micrococcus spp. and Escherichia coli.

The cell suspensions of the individual strains were prepared as in 3.2.6.1 and frozen as described in 3.2.6.2. One set of tubes were kept at -39±2°C and another set at -20±2°C. Tubes were withdrawn at definite intervals thawed rapidly at a rate of 8°C/min, Samples were serially diluted with sterile

distilled water and 0.1 ml of the inoculum spread plated simultaneously on minimal agar and trypticase soy agar (TSA). An incubation temperature of 37°C was used for E. coli, while for the other two strains, RT (29±2°C) was used. At the end of 48 h, counts were taken and the percentages of dead, injured and unharmed cells were estimated. For estimating the percentages of dead, injured and unharmed cells, the method of Straka and Stokes (1959) was followed.

To study the effect of freezing menstrua on cell injury, the experiment was repeated with n.saline, beef extract (1%, aqueous), phosphate buffer and fish muscle homogenate.

3.2.6.10 Determination of growth rate of bacteria during thawing

The bacteria given in Tables 7 and 8 were used. The experiment was performed in fish muscle medium and SWP/NB broths. The frozen tubes, stored as described in 3.2.6.2, were withdrawn at definite intervals and thawed by keeping at 29±2°C (RT), +15°C and +2°C. The counts were determined (3.2.6.3) at zero h and at intervals thereon up to 96 h. Simultaneously tubes of the unfrozen cultures of the same bacteria, in approximately same concentration, were kept to serve as control.

3.2.7 Laboratory scale studies on the bacteriology of freezing of fish and prawn

3.2.7.1 Method of preparation for freezing, freezing and frozen storage of mackerel

Mackerel for the experiment was collected from departmental vessels operating off Cochin. The material consisted of mackerel having a body weight above 85 g (large), clean, wholesome and not showing any signs of deterioration of quality (IS:6032-1971). It was immediately iced in boxes in fish to ice ratio 1:1 and brought to the laboratory within 2-4 h.

The whole lot was prepared for freezing by washing with potable water carrying 5 p.p.m. available chlorine. It was divided into 2 lots.

3.2.7.1.1 IQF freezing

Fish was frozen individually by placing in aluminium trays after wrapping in 100 gauge polythene film at -40°C in plate freezer. Freezing was complete within 120 min and the material was transferred to deep freezers maintained at -20 ± 2°C and stored for 1 year.

3.2.7.1.2 Block freezing

6 - 8 fishes were packed in duplex cartons lined inside with 100 gauge polythene paper. Sufficient chilled

potable water was added to cover the fish. Cartons were placed in aluminium trays and frozen at -40°C in contact plate freezer, for 2-3 h. The blocks were glazed by dipping in chilled potable water and were transferred to deep freezer maintained at -20±2°C and stored up to one year.

3.2.7.2 Preparation of the prawn for freezing, freezing and frozen storage

The prawn (M. dobsoni) for freezing was collected from departmental vessels operating in Cochin. The material consisted of ocean-fresh, clean, wholesome prawn (count: 100-120/kg). The material was handled in the manner outlined in IS:4303 Part II (1977). It was frozen as whole (round), headless and peeled and deveined (P & D). Details of freezing and storage are as outlined in 3.2.7.1.2 for fish.

3.2.7.3 Analysis of frozen fish/prawn

Samples were withdrawn at regular intervals and subjected to bacteriological, chemical and organoleptic tests. The total aerobic bacterial count and pathogenic indicator bacterial count was determined as per the procedures 3.2.3, trimethylamine (TMA) and total volatile nitrogen (TVN) by the method of Conway (1947), protein and non-protein nitrogen according to the method of AOAC (1975). For organoleptic evaluation, 3 to 5

fishes were taken at random, cleaned, cut to pieces in one inch dimensions, cooked in 2% brine for 15 min and assessed by taste panel experts for flavour, odour, texture and rancidity using scalar system of scoring. Maximum score assigned to each quality was 5. The scoring rate was as 5-Good, 4-Good to fair, 3-fair, 2-fair to poor, 1-poor and o-off.

3.2.8 Thawing of frozen fish/prawn

The frozen material (fish/prawn) was placed in water-proof poly thene bags put in running water at 29 ± 2°C and allowed to thaw at a rate of 2°C/min, until the individual pieces were separated and ice was completely melted. Bacteriological changes were followed by taking the bacterial count immediately before thawing and at definite intervals thereafter using the procedure outlined in 3.2.3.1. Studies were also repeated at 4°C and 15°C.

Bacterial colonies were isolated and identified as in the case of fresh fish.

3.2.8.1 Determination of the spoilage capacity of the isolated cultures

The cultures were tested for spoilage capacity by the following reactions, the details of which are given elsewhere in the thesis (3.2.5.2)

i. Proteolytic activity as judged by the ability to hydrolyse gelatin and casein.

ii. Production of hydrogen sulphide, TMA, phosphatase, Nuclease, NH_3, indole etc.

The reactions were studied at three temperatures namely, 4°C, 15°C and 29±2°C (RT).

The behaviour of these cultures in fish press juice was also studied. The fish press juice was prepared by the method of Lerke et al. (1963) as modified by Lerke et al. (1965). A heavy suspension (10^7 cells/ml) was used to inoculate the tubes of the press juice. Tubes were kept at 4°C, 15°C and 29±2°C (RT). Contents were analysed for the TPC and tested for the presence of ammonia, indole, TMA and H_2S. pH and odour were also noted. Purity of the culture was ascertained by randomly isolating 10-20 colonies and studying their morphological and biochemical characteristics.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 Factors affecting the recovery of bacteria from fish and prawn

4.1.1 Effect of plating method and incubation temperature

4.1.1.1 Fresh fish and prawn

The influence of the plating methods, viz. spread plate method (SP) and pour plate method (PP) and incubation temperatures of 37°C and room temperature (29±2°C) on total aerobic plate count (TPC) of some fresh fishes and prawns were determined. A set of plates were also incubated at 5°C to determine the psychrotrophic population.

The TPC of the fresh fishes in spread plates (SWA), at room temperature, ranged from $1.7x10^5$/g to $1.21x10^8$/g. In poured plates at this temperature, the TPC was in the range of $2.0x10^5$/g to $9.21x10^7$/g of skin and muscle. On incubation of the plates at 37°C, the TPC in spread plates were in the range of $2.0x10^5$/g to $1.11x10^8$/g and in pour plates $8.7x10^4$/g to $1.02x10^8$/g of skin and muscle. The highest bacterial count was noted in spread plates incubated at room temperature and the lowest in pour plates at 37°C. The psychrotrophic count at 5°C in SWA ranged from $3.2x10^3$/g to $1.1x10^5$/g of muscle.

In the case of fresh prawn, the bacterial count in SWA at room temperature varied from $6.1x10^4$/g of muscle

to 1.41×10^6/g and in pour plates from 5.6×10^4/g to 1.34×10^6/g. On incubation of the plates at 37°C, the spread plates carried a bacterial count ranging from 5.8×10^4/g to 1.18×10^6/g and in pour plates the bacterial count was in the range of 3.2×10^4/g to 1.07×10^6/g. At 5°C, the count varied from 4.2×10^3/g to 2.1×10^5/g. The highest count was obtained in spread plates incubated at room temperature and the lowest in pour plates incubated at 37°C.

Statistical analysis of the data was done by converting the bacterial count into their log values and analysis of variance technique was used for analysis of the data (Snedecor and Cochran, 1961). The results are presented in Table 9.

It is found that for sardines, there is no significant difference in bacterial count between temperatures but difference existed between methods, spread plates giving a greater bacterial count than pour plates. For mullet, significant difference existed between temperatures, RT giving a higher count than 37°C. Neither method of plating nor temperature had any significance in the case of mackerel.

For fresh prawn, it was found that significant difference existed in bacterial count between the two

Table 9

Analysis of variance of plating method and incubation temperature for fresh fish

Fish	Source	S.S.	df	MS	f
Sardine	Total	47.9681	71	-	c
	Samples	44.1281	5	8.8256	188.18 ***
	Temperatures	0.0040	1	0.0040	0.085
	Methods	0.8828	1	0.8828	18.82 ***
	Error	2.9529	63	0.0469	-
Mullet	Total	20.4909	59	-	-
	Samples	9.1154	4	2.2788	15.45***
	Temperatures	3.6608	1	3.6608	24.819***
	Methods	0.1937	1	0.1937	1.313
	Error	7.5207	51	0.1475	-
Mackerel	Total	7.4822	23	-	-
	Samples	7.1739	5	1.4348	95.02***
	Temperatures	0.0595	1	0.0595	3.94
	Methods	0.0076	1	0.0076	1
	Error	0.2412	16	0.0151	-
Prawn *M. dobsoni*	Total	7.7779	23	-	-
	Samples	7.6152	5	1.5230	435.14***
	Temperatures	0.1008	1	0.1008	28.80***
	Method	0.0083	1	0.0083	2.37
	Error	0.0560	16	0.0035	-

*** Significant at 0.1% level

temperatures, RT and 37°C ($p < 0.001$). The count at RT was significantly greater than that at 37°C. But there was no significant difference between the two methods at 5% level of significance. Here also, the variation between samples was found to be highly significant ($p < 0.001$).

4.1.1.2 Frozen fish and prawn

For frozen fishes, the bacterial count ranged from $3x10^3$/g of skin and muscle to $1.2x10^6$/g. The highest bacterial count was noted in spread plates incubated at room temperature and the lowest corresponded to poured plates incubated at 37°C. For frozen prawn also, a similar trend was observed

The analysis of variance of the results are presented in Table 10. Regarding frozen sardine, significant difference in count was observed between the two temperatures as well as methods. Spread plates and incubation at room temperature resulted in the maximum bacterial count for frozen mackerel and frozen jew fish. For frozen prawn, there was significant difference in bacterial count between the two temperatures. The count at RT was significantly higher than the count at 37°C. However, there was no significant difference in bacterial count between the two methods at 5% level of significance. Here also, variation between samples was found to be highly significant ($p < 0.001$).

Table 10

Analysis of variance of plating method and incubation temperature for frozen fish/prawn

Frozen fish/ prawn	Source	S.S.	df	MS	f
Sardine	Total	11.8636	71	-	-
	Samples	9.9333	5	1.9866	179.46***
	Temperature	0.2351	1	0.2351	21.24***
	Method	1.0061	1	1.0061	90.89***
	Error	0.6864	62	0.0110	-
Mackerel	Total	7.3339	23	-	-
	Samples	6.0332	5	1.2066	23.66***
	Temperature	0.2924	1	0.2924	5.73*
	Method	0.1920	1	0.1920	1.80
	Error	0.8163	16	0.0510	-
Jew fish	Total	1.6288	23	-	-
	Samples	0.7593	1	0.7593	27.51***
	Temperature	0.2280	1	0.2280	8.26*
	Method	0.1450	1	0.1450	5.25*
	Error	0.4962	18	0.0276	-
Prawn *M. dobsoni*	Total	6.4718	23	-	-
	Samples	0.2180	5	1.0436	25.45***
	Temperature	0.5327	1	0.5327	12.99**
	Method	0.0656	1	0.0656	1.6
	Error	0.6555	16	0.0410	-

* Significant at 5% level
** Significant at 1% level
*** Significant at 0.1% level

It was found that in the four varieties of fish and two types of prawn, whether fresh or frozen, the peak bacterial count was noted in spread plates, incubated at room temperature. Eventhough some variations were observed by the statistical analysis, still, majority of the analyses favoured spread plates and incubation at room temperature. According to Gjerde (1976), a higher bacterial count at 37°C in comparison to room temperature for fresh fish is an indication that the fish is exposed to higher (ambient) temperature, since normally psychrotrophic bacteria predominate in raw fish.

The growth temperature of the cultures isolated from fresh and frozen mackerel and prawn are given in Tables 11 and 12 respectively. It is seen from the tables that 100% of the isolates from fresh fish/prawn at 37°C in SWP grew equally well at room temperature. But only 58% of the isolates from room temperature could grow when incubated at 37°C. Similarly, in the case of isolates from frozen fish/prawn all the isolates from incubation temperature of 37°C in TGB could grow at room temperature as well. Contrary to this, 30% of the isolates, isolated at RT in TGA failed to grow when incubated at 37°C. This showed that the upper limit of growth temperature is one reason for the reduced recovery of bacteria at 37°C. Since many of these isolates grew

Table 11

Growth temperature range of bacteria isolated from fresh mackerel and prawn

Media	Temperature of isolation °C	Number of cultures tested	Percentage of cultures growing					
			Temperature of incubation °C					
			0	10	20	30	37	45
Sea water agar	8	62	100	100	100	90	42	Nil
	30	59	36	38	69	100	58	22
	37	58	18	18	28	100	100	[illegible]
Nutrient agar	8	72	100	100	100	72	32	Nil
	30	99	36	36	62	100	70	26
	37	87	8	8	21	100	100	48

Table 12

Growth temperature range of bacteria isolated from frozen mackerel and prawn frozen at -40°C and stored at -20±2°C

Media	Temperature of isolation °C	Number of cultures tested	Percentage of cultures growing					
			Temperature of incubation °C					
			0	10	20	30	37	45
Tryptone glucose agar	8	98	100	100	100	85	43	3
	30	93	58	69	95	100	70	31
	37	100	49	55	91	100	100	30

well, they may be considered as psychrotrophs(as per the definition of Eddy, 1960). This finding, that room temperature gives highest recoveries of bacteria from fresh and frozen fish and prawn, is confirmed by the reports of Lee and Harward (1970), Nottingham et al. (1975) and Gjerde (1976).

Regarding the two methods, Carlucci and Pramer (1957) noted that number of colonies developing on pour plates were 30-40% greater than those of spread plates. They thought this to be due to the inability of the micro-aerophilic or anaerobic bacteria to grow on agar surface.

The observation of Carlucci and Pramer (1957) differed from the present finding in that pour plates yielded higher counts. However Clark (1957), Lee and Harward (1970) and Nottingham et al. (1975) reported higher recoveries of bacteria from frozen seafoods in spread plates. While Clark (1957) attributed the higher recoveries of bacteria in spread plates to the disintegration of the clumped cells by the glass rod, Lee and Harward (1970) and Nottingham et al. (1975) suggested the presence of psychrotrophs as the reason for increased recoveries in spread plates. This is because they thought that psychrotrophs could not withstand the agar

temperature used in pour plating. This is further confirmed by the work of Klein and Shenyuwu (1974) according to which, starved microbial population (which are also stressed) may show increased sensitivity to a secondary stress such as warming which may happen during pour plating. This possibility may arise especially in the case of bacterial isolates from frozen fish. But the other possibility namely, the presence of psychrotrophs, also could not be deleted.

4.1.2 Effect of diluent

The effect of diluents on the recovery of bacteria from fresh/frozen fish and prawn was investigated. The diluents used in the study were distilled water (DW), sea water (SW), peptone water (PW), normal saline (NS), phosphate buffer (PB) and quarter strength Ringers' solution (QRS). In addition, few combinations of these diluents namely, phosphate buffered saline (PBS), peptone phosphate buffer (PPB) and peptone-saline (PS) were also tried. Normal saline was used as control.

To make a comparative study of the effect of these diluents on TPC of fresh and frozen fishes, the data was analysed by using analysis of variance after converting the bacterial count into log. values. The level of significance of the variance ratios between samples and

between diluents of fresh and frozen fish/prawn are summarised in Table 13. For fresh as well as frozen fish and prawn, significant difference existed in bacterial count between the diluents in single and in combination, the significance level ranging from 5 to 0.1%. Between samples, no significant difference existed.

The least significant difference (LSD) at 5% level and the mean logarithmic counts in ascending order for fresh and frozen sardine, mackerel, P. stylifera and M. dobsoni are presented in Table 14. For fresh sardines, ORS facilitated maximum recovery of bacteria, while for mackerel normal saline (NS) was found to cause greater recovery of bacteria. In the case of prawn M. dobsoni, Quarter strength Ringers solution was found to be ideal for getting highest TPC, while for P. stylifera phosphate buffer yielded higher bacterial count.

In the case of frozen sardine, mackerel and two varieties of prawn, normal saline consistently gave highest recovery of bacteria.

For reaching the final conclusion, the overall average of the log.values of the two types of fishes and two types of prawn were determined and this is given in Table 15. This shows that for fresh fish, the highest recovery of 5.89 was obtained when ORS was used. This was

Table 13

Summary of the level of significance of variance ratio of bacterial counts between diluents and between samples from the ANOVA

	Fresh fish				Frozen fish			
	Single		Combination		Single		Combination	
	Diluent	Sample	Diluent	Sample	Diluent	Sample	Diluent	Sample
Sardine (*Sardinella longiceps*)	$p < .001$	NS	$p < .01$	NS	$p < .05$	NS	$p < .05$	NS
Mackerel (*R. kanagurta*)	$p < .05$	NS	$p < .001$	NS	$p < .001$	$p < .05$	$p < .001$	NS
M. dobsoni	$p < .001$	NS	$p < .001$	NS	$p < .001$	NS	$p < .001$	NS
P. stylifera	$p < .05$	NS	$p < .001$	NS	$p < .001$	NS	$p < .05$	NS

NS - not significant

Table 14

Least significant difference (LSD) at 5% level and mean logarithmic bacterial count(arranged in ascending order)in different diluents for fresh and frozen/prawn

	Fresh fish/prawn				Frozen fish/prawn			
	Single		Combined		Single		Combined	
		0.4834		0.0734		0.0595		0.1522
Sardine (Sardinella longiceps	PW	5.6032	PS	5.2299	QRS	4.9081	PS	4.6120
	PB	5.7089	PBS	5.2394	DW	4.9539	PPB	4.7608
	DW	5.7250	NS	5.3609	PB	4.9786	PBS	4.8343
	NS	6.4117	PPB	5.3613	NS	5.1777	NS	4.8545
	SW	6.65533						
	QRS	6.7931						
		0.4154		0.0219		0.1130		0.0489
Mackerel (Rastrelliger kanagurta)	PB	5.4606	PPB	5.5184	DW	4.8997	PPB	4.8709
	DW	5.4606	PS	4.5258	QRS	5.1110	PBS	4.9124
	QRS	5.5006	PBS	5.6642	PB	5.1514	PS	4.9680
	PW	5.6263	NS	5.6739	PW	5.2131	NS	5.0531
	SW	5.8530			NS	5.2619		

Table contd.

	Fresh prawn			Frozen prawn		
	Single		Combined	Single		Combined
	0.4156		0.0580	0.1384		0.2252
P. stylifera	DW 4.8717	PS	5.4709	QRS 4.5853	PBS	4.1079
	NS 5.1889	PPB	5.6472	PB 4.8018	PPB	4.1996
	SW 5.2022	NS	5.7573	DW 5.0040	NS	4.3988
	QRS 5.2811	PBS	5.7788	PW 5.0970	PS	4.5252
	PW 5.6269			NS 5.2056		
	PB 5.6431					
	0.2827		0.0200	0.0802		0.0894
M. dobsoni	PW 4.3127	PS	6.7222	DW 5.2005	PPB	5.5382
	DW 4.3358	PPB	6.7262	PB 5.2450	PBS	5.5432
	SW 4.4431	PBS	6.8707	QRS 5.4408	PS	5.6756
	QRS 4.4877	NS	6.9711	NS 5.5962	NS	5.46994
	NS 4.5481					
	PB 4.6073					

closely followed by NS, the average log value being 5.88. In the case of frozen fish and prawn, the highest value was with NS, being 5.30. None of the combination of the diluents came up to this level.

This shows that eventhough the recovery of bacteria in different diluents varied slightly according to the sample, the recovery of bacteria in n.saline was the highest in majority of cases. This is also supported by Table 16, which shows the recovery of individual isolates from fish using these diluents. For Pseudomonas sp. Moraxella sp., Flavobacterium sp., Micrococcus sp. and Bacillus sp., the recovery was highest when n.saline was used as diluent. But, for E. coli, phosphate buffer caused maximum recovery while in the case of Acinetobacter sp. peptone water facilitated maximum count.

Straka and Stokes (1957) recommended 0.1% peptone water as diluent for Pseudomonas, E. coli and S. fecalis But according to Jayne Williams (1963), double distilled water and phosphate buffer was found to be ideal diluent than 0.1% peptone water for Pseudomonas aeruginosa, Streptococcus fecalis and E. coli. Hoadley and Cheng (1974), while studying the recovery of Pseudomonas aerigenosa, Streptococcus fecalis and E. coli, showed that tap water was highly toxic to all the strains. While the recovery of P. aeruginosa was most successful when

Table 15

Average log,value of bacterial count at RT in SWA

Diluent	Fresh fish		Frozen fish	
	Single	Combination	Single	Combination
DW	5.33	-	5.01	-
SW	5.84	-	-	-
NS	5.88	-	5.30	-
PW	5.62	-	5.25	-
PB	5.60	-	5.07	-
QRS	5.89	-	5.01	-
PS	-	5.24	-	4.69
PPB	-	5.66	-	4.75
PBS	-	5.33	-	4.60
NS	-	5.67	-	4.60

Table 16

Recovery of bacterial strains on SWA at RT in various diluents(bacterial count/ml)

Bacterial species	Distilled water	Sea water	Normal saline	Peptone water	Phosphate buffer	
Pseudomonas sp.	3.8×10^{6}*	7.6×10^{6}	7.7×10^{6}	5.3×10^{6}	6.1×10^{6}	5.6×10^{6}
Acinetobacter sp.	3.2×10^{7}	3.6×10^{7}	3.3×10^{7}	4.1×10^{7}	1.2×10^{8}	7.5×10^{6}
Flavobacterium sp.	1.1×10^{6}	2.2×10^{6}	4.2×10^{6}	2.7×10^{6}	3.1×10^{6}	3.0×10^{6}
E. coli	3.5×10^{8}	3.6×010^{8}	4.4×10^{8}	3.9×10^{8}	5.2×10^{8}	4.1×10^{8}
Micrococcus sp.	7.21×10^{7}	7.5×10^{7}	8.1×10^{7}	7.3×10^{7}	7.7×10^{7}	7.81×10^{7}
Bacillus sp.	8.03×10^{8}	8.31×10^{8}	8.8×10^{8}	8.11×10^{8}	8.23×10^{8}	8.19×10^{8}
Moraxella sp.	3.7×10^{7}	8.2×10^{7}	8.8×10^{7}	7.3×10^{7}	6.8×10^{7}	6.4×10^{7}

*Average of 3 values

phosphate buffer was used, it had no effect on improving the count of Escherichia coli and S. fecalis.

Microbial counts of frozen raw shrimp and processed shrimp, obtained by using the diluents Butter field's phosphate buffer and 0.2% peptone, showed the former to be superior as a diluent (Lee and Harward, 1970). Sinnhuber and Lee (1964) also recommended Butterfield's phosphate buffer to be used as diluent for determination of TPC of irradiated seafoods.

The diluent effect of bacteria may be considered to be osmotic or ionic. But, study of Gray et al. (1977) suggested that the diluent effect was ionic and not osmotic. According to them, cations enhanced the protective property of the diluent. This finding is further substantiated by the report of Devoc and Oginsky (1969) that marine bacteria require cations to maintain integrity of cell wall by shielding anionic groups and thus stabilising cell envelope.

4.1.3 Effect of time of holding the dilution on the survival of bacteria

The effect of holding the inoculum for different periods in the dilution fluids distilled water (DW), sea water (SW), phosphate buffer (PB), and n.saline (NS) has been studied and figures 1 and 2 represent the variation

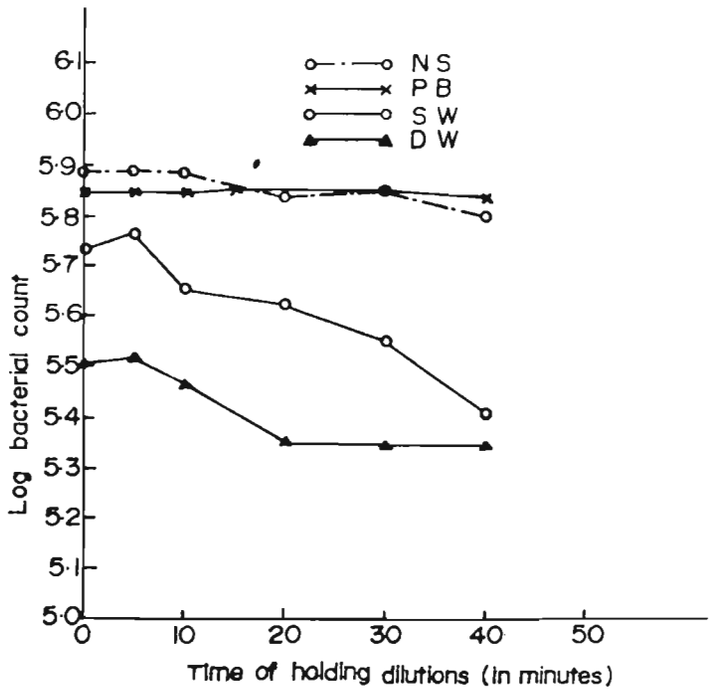

Fig.1.
EFFECT OF HOLD UP TIME ON THE SURVIVAL AND RECOVERY OF BACTERIA FROM FRESH FISH IN DIFFERENT DILUENTS.

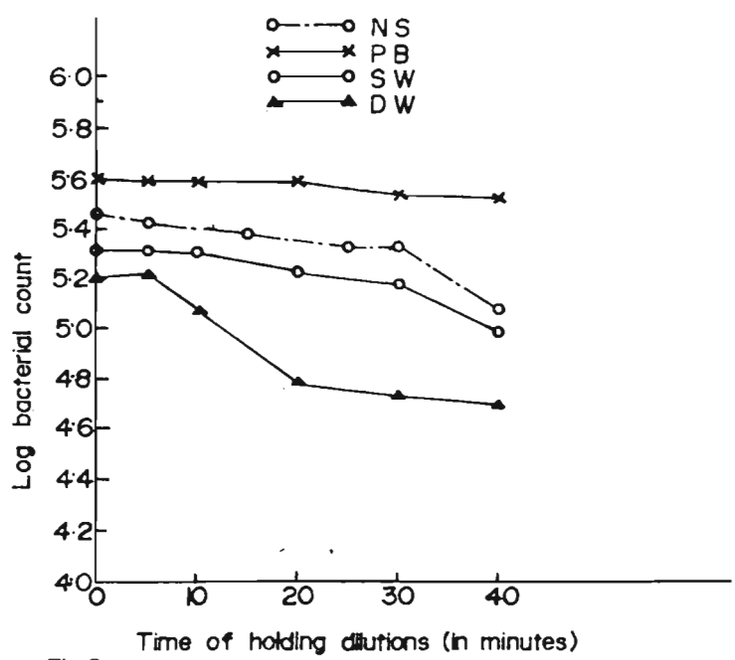

Fig.2.
EFFECT OF HOLD UP TIME ON THE SURVIVAL AND RECOVERY OF BACTERIA IN FROZEN FISH IN DIFFERENT DILUENTS.

in bacterial count on keeping the inoculum from fresh and frozen fish/prawn in these diluents (ie. hold-up time). Figures 3 and 4 show the typical data on effect of holding time of pure cultures of bacteria in the diluents.

The time lag required for preparation of the inoculum was adjusted to be identical to all the samples and did not exceed 7 minutes. The zero hour count represented the bacterial count of the sample immediately after adding the inoculum to the working dilution of the diluent.

As seen from the figure for fresh fish (Fig.1), phosphate buffer and n.saline gave more or less a steady count up to holding for 30 minutes. After holding the dilution for 40 minutes, the bacterial count in saline started to go down, while in phosphate buffer it remained stationary. Sea water, after a transient increase in count corresponding to 5 minutes, noted a fall. In distilled water, there was a continuous fall in count.

For frozen fish, holding the inoculum in phosphate buffer produced steady counts up to 40 minutes, whereas in n.saline the bacterial count came down after 30 minutes. The bacterial count in phosphate buffer was more stable than n.saline. Sea water showed a gradual

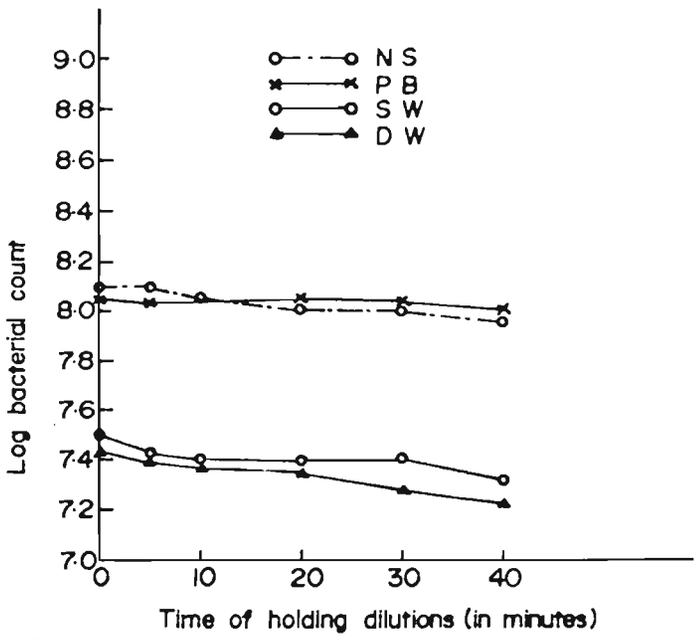

Fig.3.
EFFECT OF HOLD UP TIME ON THE SURVIVAL AND RECOVERY OF PSEUDOMONAS STRAIN IN DIFFERENT DILUENTS.

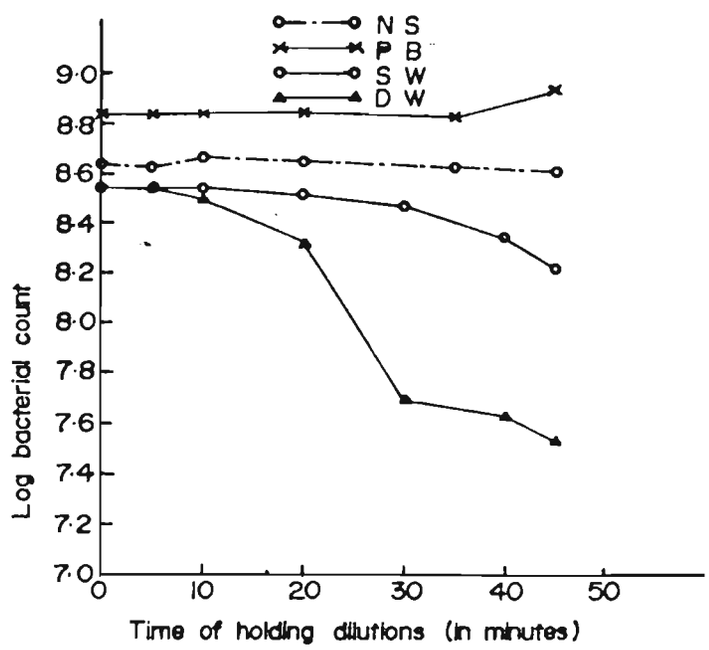

Fig.4.
EFFECT OF HOLD UP TIME ON THE SURVIVAL AND RECOVERY OF AN ESCHERICHIA COLI STRAIN IN DIFFERENT DILUENTS.

decrease in bacterial count up to 30 minutes. In distilled water, more severe fall in bacterial count was noted in the first 30 minutes.

Figure 3 shows the effect of holding pure culture of Pseudomonas in the four diluents. Holding the dilution in n.saline and phosphate buffer produced steady counts up to 30 minutes of holding time after which the bacterial count fell slightly. In sea water, a decrease was noted in bacterial count after holding for 20 minutes. But suspending the cells in distilled water produced steady counts up to 30 minutes.

When the Escherichia coli cells were suspended in phosphate buffer (Fig.4), the count remained steady up to 30 minutes after which an increase was noted. This increase could be attributed to multiplication of cells.

Thus, it is clear that for fresh/frozen fish diluents like n.saline and phosphate buffer registered almost uniform bacterial count even up to 30 minutes in addition to giving high recoveries. This was true for two pure cultures of bacteria also. Distilled water and sea water caused poor recoveries of bacteria in the samples. Antibacterial effect of sea water (Carlucci and Pramer 1959) or cellular damage caused by distilled water (Zaske et al., 1980) may be the reason.

According to Jayne Williams (1963), "the function of a diluent is to enable a true assessment to be made of condition of bacterial population and death or revival of organisms should not take place during the dilution process". It is seen that these conditions have been strictly followed by the diluents n.saline and phosphate buffer studied here.

4.1.4. Effect of period of incubation on the recovery of bacteria

4.1.4.1 Fresh fish and prawn

The observed increase in total plate count after 24, 48, 72 and 96 hours of incubation in SWA was studied.

The data were pooled and the total bacterial count was converted to the log. values which were subjected to statistical analysis by analysis of variance technique. The statistical analysis is presented in Table 17. The data indicates that for fresh fish and prawn, there is significant difference ($p < 0.01$) in bacterial count at the four incubation periods. The least significant difference at 1% level for the logarithm of bacterial count was 0.2558 and mean logarithmic increase in count after 24, 48, 72 and 96 hours of incubation were respectively 2.4906, 2.3310, 1.7868 and 1.5365.

Table 17

Analysis of variance of bacterial count at four incubation periods in fresh fish/prawn

Source	S.S.	df	ms	F
Total	16.5691	47	-	-
Incubation periods	7.3871	3	2.4624	46.90**
Samples	7.4511	11	0.6774	12.90**
Error	1.7309	33	0.0525	

Table 18

Analysis of variance of bacterial count at four incubation periods in frozen fish/prawn

Source	S.S.	df	ms	F
Total	13.5903	47	-	-
Incubation periods	6.0294	3	2.0098	56.61**
Samples	6.3904	11	0.5809	16.36**
Error	1.1705	33	0.335	-

**Significant at 1% level

There is no significant difference in count between 24 and 48 hours of incubation. Similarly, the coefficient of variation of the count at the four incubation periods were 14.24%, 20.33%, 27.98% and 31.88% respectively. The variations between samples were significant at 1% level.

4.1.4.2 Frozen fish and prawn

Like fresh fish/prawn, frozen fish also showed significant difference ($p < 0.01$) in the increase in bacterial count after the four periods of incubation viz. 24, 48, 72 and 96 hours (Table 18). The least significant difference in this case was 0.2104 and the mean logarithmic count after 24, 48, 72 and 96 hours of incubation were 1.5489, 1.9231, 1.3651 and 0.9377 respectively. The increase in bacterial count after 48 hours was significantly high compared to the other three. The coefficient of variation in the bacterial count after the four periods of incubation were 28%, 19.08%, 27.86% and 50.11% respectively. The variation between sample was significant at 1% level.

4.1.5 Effect of composition of the medium

The effect of medium composition on the quantitative and qualitative recovery of bacteria, is discussed under succeeding chapters. Hence, that part is not included here.

4.2 Bacteriology of newly caught mackerel (Rastrelliger kanagurta) and prawn (Metapenaeus dobsoni)

4.2.1 Bacteriology of newly caught mackerel

4.2.1.1 Quantitative aspects

Total plate count (TPC) of newly caught mackerel (Rastrelliger kanagurta) in two plating media viz. SWA and TGA at three temperatures of incubation are presented in Table 19.

The skin with muscle, gills and intestine of newly caught mackerel registered total plate counts in the range of $6.3x10^3$ to $8.3x10^6$/g, $8.9x10^4$ to $5.6x10^6$/g and $5.5x10^4$ to $6.2x10^7$/g respectively on SWA on incubating at room temperature. The corresponding values on TGA were $8.9x10^3$ to $4.1x10^5$/g, $2.4x10^3$ to $6.3x10^6$/g, $3.2x10^3$ to $5.4x10^6$/g respectively. These results showed that intestine carried the greatest number of bacteria. This was followed by gills and the skin with muscle carried the lowest bacterial populations. This pattern was found to occur at all the three temperatures of incubation. Also, the recovery of bacteria was comparatively less on TGA than SWA. In the case of skin with muscle, only 7% of the total plate count on SWA, was recovered on TGA at RT. Similarly, for gills, 11.4% of the TPC on SWA at RT was recovered on TGA and for intestine 21.6% of the

Table 19

Total plate count of freshly caught mackerel Rastrelliger kanagurta[I]
(Total plate count/g)

Temperature of incubation °C	Count on SWA			Count on TGA		
	Skin and muscle	Gills	Intestine	Skin and muscle	Gills	Intestine
5	1.2×10^4 - 1.3×10^5 (6.3×10^4)	1.0×10^4 - 3.2×10^5 (9.5×10^4)	4.6×10^3 - 9.1×10^5 (7.3×10^4)	6.2×10^3 - 3.1×10^4 (9.4×10^3)	8.4×10^3 - 5.9×10^5 (1.1×10^3)	9.8×10^2 - 3.2×10^5 (2.1×10^3)
RT (29±2°C)	6.3×10^3 - 8.3×10^6 (1.2×10^5)*	8.9×10^4 - 5.6×10^6 (6.4×10^6)	5.5×10^4 - 6.2×10^7 (2.4×10^7)	8.9×10^3 - 4.1×10^5 (8.5×10^3)	2.4×10^3 - 6.3×10^6 (7.3×10^5)	3.2×10^3 - 5.4×10^6 (5.2×10^6)
37	3.1×10^3 - 5.6×10^4 (8.4×10^4)	2.0×10^4 - 3.3×10^7 (1.4×10^6)	5.9×10^4 - 9.2×10^7 (3.6×10^7)	7.4×10^3 - 4.4×10^5 (8.2×10^4)	1.1×10^4 - 6.2×10^6 (8.4×10^5)	4.2×10^3 - 8.4×10^6 (8.2×10^6)

*Average count of 2 trials with triplicate plates

I - studies conducted during the period 1982-1984

TPC on SWA at RT could be obtained on TGA (Table 19). In SWA, maximum bacterial count of the skin with muscle and gills were obtained at room temperature while intestine with content showed peak value at 37°C. This showed that intestine carried greater number of bacteria having their optimum growth at 37°C ie. mesophiles. The TPC at 5°C was the least compared to the counts at RT and 37°C, for skin and muscle, gills and intestine with contents. This showed that the population of the psychrotrophic bacteria was very small in all the three parts of the fish.

The bacterial count reported by earlier workers for freshly caught marine fish agrees well with the data presented in Table 19. Thus the bacterial count reported for oil sardine, Sardinella longiceps at RT on SWA was in the range of 10^3 - 10^7/g according to Karthiayani and Iyer (1967) and Surendran and Gopakumar (1982). For [illegible] mackerel, the latter reported counts in the range of 10^4 - 10^6/g. For Australian mullet, Gillespie and Macrae (1975) observed a TPC of $10^4/cm^2$ on the skin surface. The data compiled by Limados Santos (1980) for tropical fish showed the TPC in the range of 10^3 - 10^7 organisms/cm^2 of skin. This is higher to the range of 10^2 - 10^5 bacteria/cm^2, reported for cold water fish species by Shewan (1977) and Shewan and Hobbs (1967).

The peak counts of bacteria, when incubated at room temperature, have been noted previously by Karthiayani and Iyer (1967), Anand and Setty (1977) and Surendran and Gopakumar (1982) for marine fish from tropical seas. Contrary to this, the maximum TPC reported for temperate water fish was at an incubation temperature of 20°C (Shewan, 1977). This again points to the mesophilic character of the bacteria present in tropical fishes.

4.2.1.2 Qualitative aspects

The generic distribution of bacteria on skin with muscle, gills and intestinal contents of mackerel is presented in Table 20. Even though both SWA and TGA were included for the quantitative studies, the qualitative studies were undertaken with cultures isolated from SWA alone. This was on the basis of the general performance of SWA as a recovery medium.

The skin with muscle of freshly caught mackerel when plated on SWA and incubated at room temperature showed a predominance of Vibrio spp. (30%). This was followed by Pseudomonas spp. (23%), Acinetobacter spp. (14%) and Moraxella spp. (11%). The Gram-negatives thus accounted for 89% of the total bacteria, the rest being constituted by Gram-positives. The members of the Gram-positive group were Micrococcus, Bacillus and Arthrobacter. The

Table 20

Distribution of various microbial groups* in different parts of the mackerel *R. kanagurta*

Bacterial species	Percentage of the total isolates isolated from								
	Skin & muscle			Gills			Intestine		
	Incubation temperature °C								
	5	RT	37	5	RT	37	5	RT	37
Pseudomonas									
Group I	3+	1	0	3	1	0	0	0	0
Group II	10	8	9	12	10	10	3	2	2
Group III	5	6	4	10	8	8	3	3	4
Group IV	8	8	7	12	8	8	24	13	11
Moraxella	10	11	12	13	10	11	26	20	16
Acinetobacter	12	14	15	12	12	10	7	8	11
Vibrio	26	30	32	22	30	31	10	21	24
Flavobacterium/ cytophaga	8	8	7	6	7	6	2	3	4
Alcaligenes	4	2	4	1	3	2	0	0	0
Aeromonas	1	1	1	1	2	2	1	1	1
Photobacterium	3	0	0	0	1	0	1	2	2
Enterobacteriaceae	0	1	2	0	0	0	0	1	1
Micrococcus	1	6	3	3	4	6	2	8	8
Bacillus	1	2	2	1	2	4	1	4	2
Arthrobacter	6	2	2	2	3	3	10	10	12
Unidentified	0	0	2	2	0	1	4	4	2
Total	87	93	86	96	103	93	84	91	76

*Isolated from SWA; +Expressed as % of total isolates

decrease in the incubation temperature from 37°C to 5°C resulted in an increase in the proportion of Pseudomonas from 20% to 26% and a decrease in the proportion of Vibrio species from 32% to 26% in the case of skin and muscle.

The gills harboured more or less similar flora as the skin with muscle. However, the percentage at RT of Pseudomonas was slightly higher (27%) in gills compared to skin with muscle (23%).

The various bacterial species present in the intestine with contents were same as the skin flora; however, their relative proportions differed. Vibrio species constituted 21% of the intestinal flora, Pseudomonas 18% and Moraxella, 20%.

The study showed that the incubation temperature had a significant influence only on certain species, while others were unaffected. The Pseudomonas were recovered in greater numbers by decreasing the temperature of incubation, while the Vibrio species diminished in number when temperature was lowered. The other Gram-negative groups showed more or less equal distribution at the three temperatures of incubation (Table 20).

The early works of Venkataraman and Sreenivasan (1952, 1954) showed a high incidence of Gram-positives

especially, Bacillus in the slime and gills of Indian mackerel (Scomber sp.) from Calicut. But Jadhav and Magar (1970) reported a different flora predominated by Achromobacter, Bacillus and Micrococcus, for mackerel, (Rastrelliger kanagurta) from Bombay coast. These reports differed from that of Karthiayani and Iyer (1967) and Surendran and Gopakumar (1982) in having a lower incidence of Gram-negatives. Karthiayani and Iyer (1967) reported Achromobacter group (32%) as the major bacterial species in oil sardine (Sardinella longiceps). This was followed by Vibrio species which formed 27% of the total. Banik et al. (1976) found a preponderance of Flavobacterium and Micrococcus species for fresh mackerel, R. kanagurta. The same species were reported for some marine fishes from Mangalore coast (Devaraju and Setty, 1985). Vibrio species have been found to constitute the bulk of the flora of sardine (Sardinella longiceps) and mackerel (R. kanagurta) according to the report of Surendran (1980).

The present study confirms the earlier finding of Surendran (1980) in having a dominance of Vibrio species in mackerel (R. kanagurta) caught off Cochin waters. It is also in agreement with the finding of Karthiayani and Iyer (1967) for oil sardines provided the Achromobacter group is considered to be composed of the genera Moraxella

and Acinetobacter as done in the present investigation. Thus the bacterial flora reported for cold water fish species fit well with the present study, excepting the case of Vibrio. The cold water fishes carried negligible portion of vibrio species.

According to Horsley(1977), the high incidence of Bacillus sp. reported may be due to seasonal variation, geographic location and fish habitat, particularly the feed. The high incidence of Micrococcus should be ascribed to their population density or contamination from handling (Horsley, 1977). Since the latter possibility is ruled in freshly caught fish, the high initial density in water from sediment matter may be the reason.

4.2.2 Bacteriology of freshly caught prawn, Metapenaeus dobsoni

4.2.2.1 Quantitative aspects

Quantitative aspects of the bacterial flora of the muscle and the intestine of the marine prawn (Metapenaeus dobsoni) are presented in Table 21. The effects of the recovery media namely, SWA and TGA and the temperatures namely, 5°C, RT (29±2°C) and 37°C are also presented.

The average TPC of the muscle of the prawn on SWA was 8.6×10^6/g (range: 9.1×10^3 to 8.2×10^7/g) at room temperature, 7.1×10^6/g (range: 5.6×10^3 to 7.2×10^7/g) at

Table 21

Total bacterial count of the muscle and intestine of freshly caught prawn *Metapenaeus dobsoni*[I] (total plate count/g)

Temperature of incubation °C	Count on SWA		Count on TGA	
	Muscle	Intestine	Muscle	Intestine
5	1.1×10^{3} - 3.2×10^{6} *(6.3×10^{5})	1.16×10^{4} - 7.5×10^{7} (4.5×10^{5})	4×10^{2} - 5.1×10^{6} 5.0×10^{5})	9.8×10^{6} - 6.4×10^{7} (3.1×10^{5})
RT (29±2°C)	9.1×10^{3} - 8.2×10^{7} (8.6×10^{6})	3.94×10^{6} - 1.2×10^{8} (2.3×10^{7})	6.1×10^{3} - 7.4×10^{5} (6.4×10^{6})	4.0×10^{6} - 1.3×10^{8} (2.2×10^{7})
37	5.6×10^{3} - 7.2×10^{7} (7.1×10^{6})	5.1×10^{6} - 1.3×10^{8} (4.2×10^{7})	5.2×10^{3} - 9.4×10^{6} (5.5×10^{5})	4.7×10^{2} 1.28×10^{8} (5.1×10^{7})

* Average count of three trials with triplicate plates

I. studies were conducted during the period 1982-84

37°C, 6.3×10^5/g (range: 1.1×10^3 to 3.2×10^6/g) at 5°C. This showed that for prawn, as in the case of mackerel, the peak values were recorded on incubation at room temperature. The bacterial count at 5°C was 7.3% of count at RT on SWA and 7.8% of the count at RT on TGA.

While the intestine had higher bacterial counts at 37°C than at RT, the reverse was observed for the muscle. In this respect also the behaviour of bacteria from prawn was similar to that from mackerel.

The effect of the composition of the culture media reflected in the total plate counts. SWA was found to recover greater numbers of bacteria from prawn and the count difference between the two media was one log cycle in some instances.

The average bacterial counts reported for prawns from tropical sea ranged from 10^3 to 10^7/g of muscle (Liston, 1980). Sreenivasan (1959) observed a TPC of 1.82×10^6/g for fresh prawn. According to the study of Surendran (1980), the TPC of prawn P. indicus at RT ranged from 10^2 to 10^5/g, that of M. dobsoni from 10^3 to 10^6/g and M. affinis from 10^2 - 10^6/g. Most of the data available on tropical prawn from India pertains to the freshly landed material (Pillai et al., 1961; Jacob et al., 1962; Lakshmanan et al., 1984).

The TPC of the shrimp from Thailand was found to be in the range of 10^3 to 10^6/g (Cann, 1971). Most of the samples of shrimp, studied in Northern Australia, were found to harbour a bacterial load in the range of 10^3 to 10^5/g (Ruello, 1974).

There is a lot of data available on the bacterial population of shrimps from colder waters of the world. Green (1949) found bacterial counts of 1.6×10^3 to 1.6×10^5 per gram for fresh shrimp caught from Gulf of Mexico with an average count of 4.2×10^4/g. Fieger (1950) and Fieger et al. (1956) reported TPCs of 10^4 to 10^7/g at 20°C for shrimp from Gulf of Mexico. The total bacterial count of fresh prawn, (Pandalus jordani) according to the investigations of Harrison and Lee (1969), ranged from 4.2×10^3 to 4.4×10^3/g muscle. The TPC observed for Scampi, (Nephrops sp.) was 10^3 to 10^6/g, according to the report of Walker et al. (1970) and 10^4/g, according to the report of Hobbs et al. (1971). A TPC in the range of 10^4 to 10^7/g was recorded for deep water shrimp, Pandalus borealis and 10^4 to 10^5/g for inshore shrimp, Pandalus montagui and Crangon crangon (Cann, 1977). For pacific shrimp, Pandalus jordani, Lee and Pfeifer (1977) noted a total count of 1.6×10^5/g to 3.2×10^6/g of meat.

The results presented in Table 21 clearly showed that the TPCs of the tropical shrimp (M. dobsoni), when freshly caught, compares well with that of cold water shrimp.

4.2.2.2 Qualitative studies

The generic distribution of bacteria on the muscle and intestine of the freshly caught prawn Metapenaeus dobsoni are presented in Table 22. The data also shows the effect of incubation temperatures viz. 5°C, RT (29±2°C) and 37°C on the qualitative recovery of bacteria.

The Acinetobacter - Moraxella group (which was formerly grouped as Achromobacter) was the most predominant bacterial species found in muscle of the freshly caught prawn. The recovery of this group at 5°C, RT and 37°C were respectively 39%, 37% and 37%. Between the two genera, the proportion of Moraxella was greater than that of Acinetobacter.

The Acinetobacter - Moraxella group was followed by Pseudomonas and Vibrio species which at RT, constituted 20% of the flora. At 37°C the percentage of Vibrio was 22 and that of Pseudomonas, 15. On incubation at 5°C, the percentage of Vibrio and Pseudomonas came to 16 and 20 respectively. The Flavobacterium sp. showed lower incidence viz. 6%, 9% and 7% at incubation temperature of RT, 37°C and 5°C. These microbiol groups constituted

Table 22

Distribution of various microbial groups* on muscle and intestine of prawn M. dobsoni

Bacterial species	Percentage of total isolates isolated from					
	Muscle			Intestine		
	Incubation temperature °C			Incubation temperature °C		
	5	RT	37	5	RT	37
Pseudomonas	20+	20	15	10	8	6
Moraxella	23	22	19	14	13	9
Acinetobacter	16	15	18	18	16	12
Vibrio	16	20	22	16	20	22
Flavobacterium/ cytophaga	7	6	9	10	11	12
Alcaligenes	1	1	1	1	0	1
Enterobacteriaceae	0	0	0	0	0	0
Micrococcus	5	4	5	13	14	16
Bacillus	4	2	3	8	11	13
Arthrobacter	8	10	9	9	7	9
Unidentified	0	0	0	1	0	0
Total isolates	97	100	98	92	98	93

*Isolated from SWA; + Expressed as percentage of total isolates

more than 80% of the total flora showing that bulk of the flora of the prawn muscle was Gram-negative in nature. Thus the effect of incubation temperature was not very significant in this case.

In the intestine with contents also, the predominant bacterial group was Acinetobacter - Moraxella spp. The percentage of this group at 5°C, RT and 37°C were respectively 21, 29, 32. While the Vibrio species were found to constitute 20% of the flora at RT, the Pseudomonas were less constituting only 8% of the flora. The Flavobacterium species (11%) at RT were slightly higher than in muscle. The Flavobacterium species constituted bulk of the flora of intestine of prawn P. indicus according to the investigation of Bose and Chandrasekaran (1976).

The Gram-positives together constituted 25% of the flora, Micrococcus accounting for 11%, Bacillus 7%, rest being Arthrobacter. Their share is slightly on the higher side when compared with the muscle of prawns.

With regard to the tropical prawn from Bombay (P. stylifera), Shaikmahmud and Magar (1956) noticed a greater proportion of Acinetobacter, followed by Bacillus, Micrococcus and Pseudomonas groups. A preponderance of Micrococcus and Coryneforms were encountered for different

varieties of prawn from the same area by Sreenivasan (1959).

According to a report of Bose (1969), Vibrio constituted large proportion of the bacterial flora of tropical prawn. Bose and Chandrasekaran (1976) reported Vibrio species (28%) in slime of prawn caught off Nagapattinam near Madras. Achromobacter constituted 22% of the flora, Pseudomonas 10% and Flavobacterium and Micrococcus, 15% each.

The bacterial flora reported for shrimp from temperate and cold waters show similarities with that reported in this study. For pacific shrimp, Pandalus jordani the microbial flora in raw shrimp in the order of predominance was Acinetobacter - Moraxella, Flavobacterium, Pseudomonas Gram-positive cocci and Bacillus (Harrison, and Lee, 1969). Williams et al. (1952) found the main bacterial groups in the Gulf shrimp to be Achromobacter, Micrococcus, Pseudomonas and Bacillus. High incidence of Achromobacter, followed by Coryneforms and Flavobacterium was noted for shrimp Pandalus borealis, Pandalus montagui and Crangon crangon from North sea (Cann, 1977). But in another report, Pandalus borealis caught from the same area had a very high incidence (63%) of Corynebacterium (Walker et al., 1970). High incidence of Coryneforms (81%) in Gulf shrimp have been reported by Vanderzant et al.

(1971). Lee and Pfeifer (1977) reported that microorganisms associated with Pacific shrimp, Pandalus jordani in the order of dominance were Moraxella, Pseudomonas, Acinetobacter and Flavobacterium/Cytophaga.

The present study shows that the Acinetobacter-Moraxella group together formed the bulk of the flora of the tropical prawn, M. dobsoni. Of the different bacterial strains Moraxella formed the highest proportion followed by Pseudomonas, Vibrio and Acinetobacter. It can be seen that the major difference in the flora of tropical prawn from that of cold water species is in the proportion of Vibrio. Otherwise, the flora compares well with that of Cann (1977), Lee and Pfeifer (1977), and Harrison and Lee (1969).

4.3 Effect of low temperature on bacteria associated with fish/prawn

4.3.1 Survival of bacteria associated with fish/prawn

4.3.1.1 Survival of some marine bacteria isolated from fish/prawn

Pure cultures (4 to 6 strains) of bacteria, representing the common genera isolated from fish/prawn, were subjected to in-vito studies. The bacterial cultures were exposed to various temperatures viz. $7\pm1°C$, $-20\pm2°C$ and $-39\pm2°C$ in two freezing menstrua

viz. fish muscle medium(FMM) and sea water peptone broth (SWP). These temperatures corresponded to the refrigeration, frozen storage and freezing temperatures.

The survival curves of the typical strains of each bacterial genera at the three temperatures are given in figures 5 to 9. The survival curves were plotted by taking the logarithm of the number of survivors on 'X' axis and period of storage in days on the 'Y' axis.

The survival curves at -39±2°C and -20±2°C were of almost identical pattern. There was a steep slope in the curve which corresponded to the first 24-48 h of storage. This was followed by a curve of lesser slope and was found to occur within the first 10 days. After this there was very little change and the curve was almost straight in some cases. This general pattern was found to exist in all the Gram-negative bacteria. In the Gram-positive Micrococcus spp. however, there was a short drop in the curve in the early period after which it was almost straight line. The first phase may be due to effect of freezing while the other two could be ascribed to frozen storage. The curve also pointed to the fact that the largest part of decline in the cell population occurred during the first 10 days or more precisely in the first day.

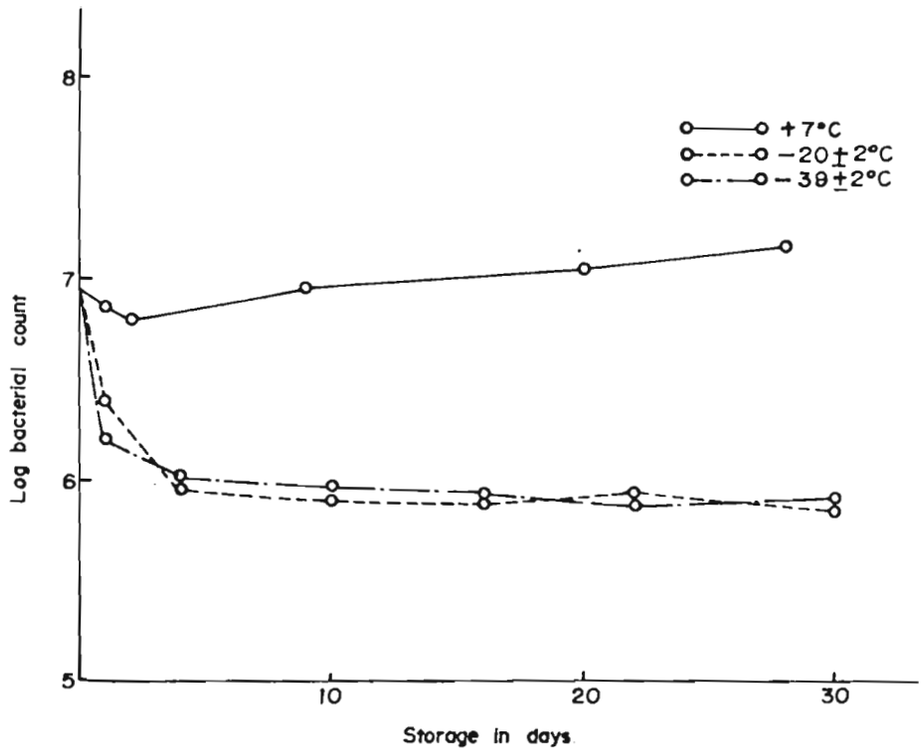

Fig.5.1.
SURVIVAL OF A PSEUDOMONAS STRAIN IN FISH MUSCLE MEDIUM AT DIFFERENT TEMPERATURES

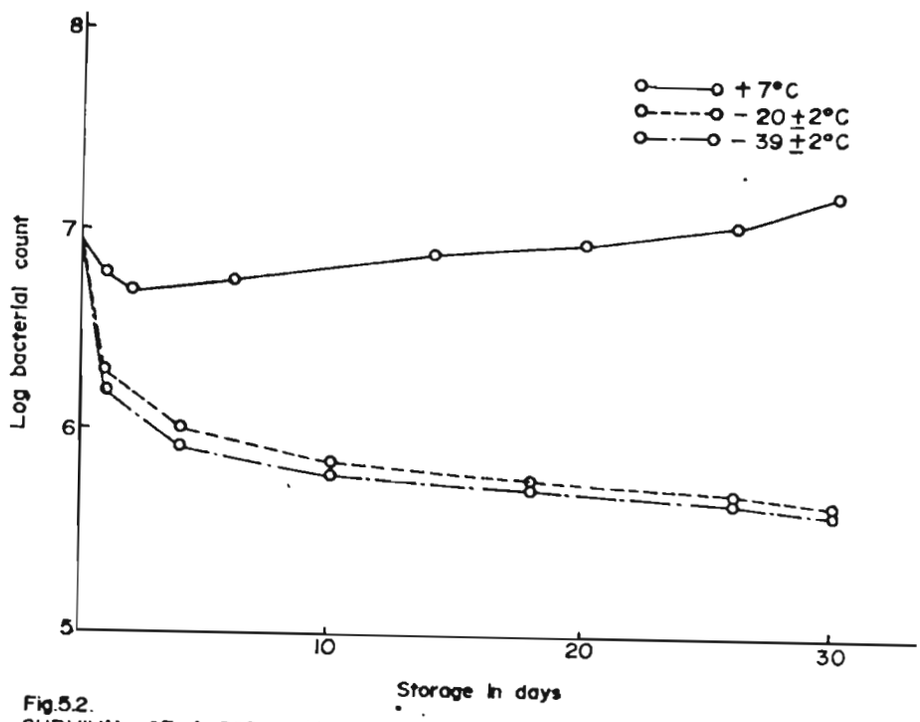

Fig.5.2.
SURVIVAL OF A PSEUDOMONAS STRAIN IN SWP BROTH AT DIFFERENT TEMPERATURES

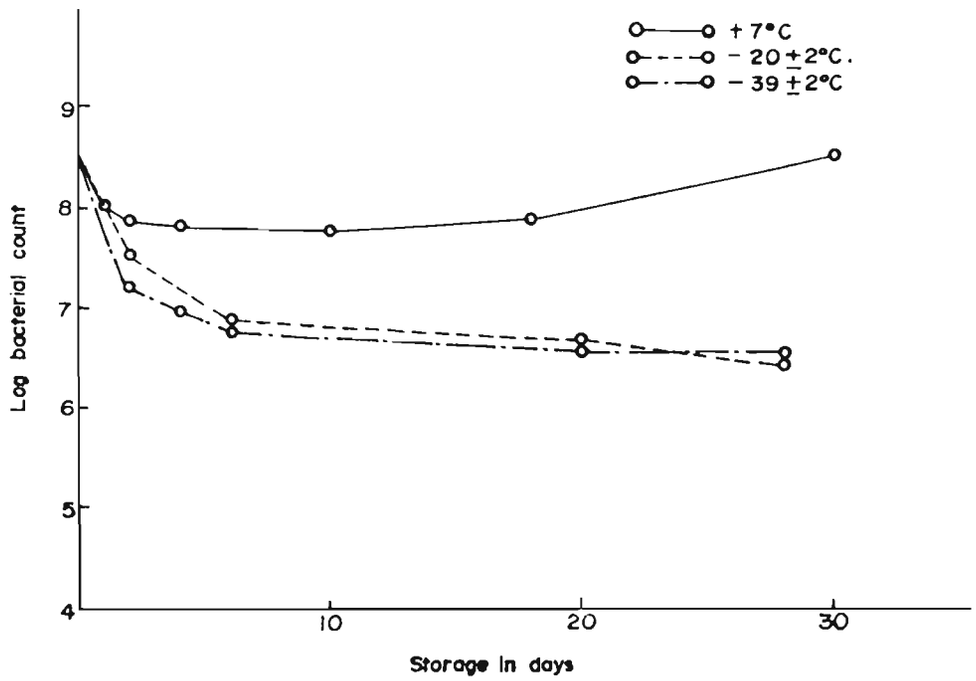

Fig.6.1.
SURVIVAL OF A MORAXELLA STRAIN IN FISH MUSCLE MEDIUM AT DIFFERENT TEMPERATURES

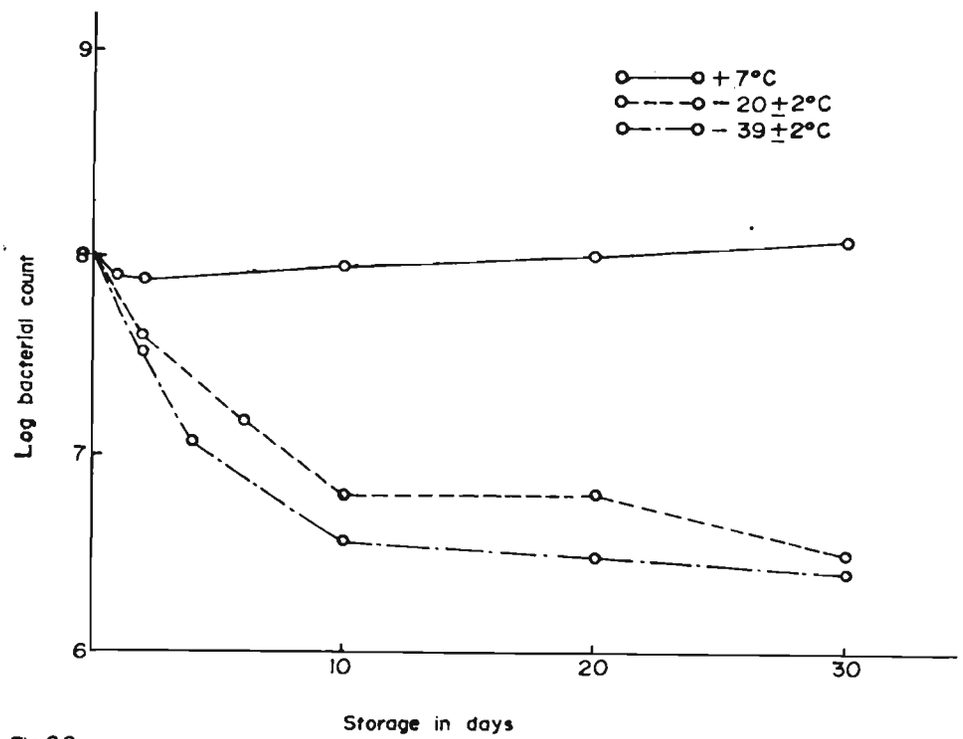

Fig.6.2.
SURVIVAL OF A MORAXELLA STRAIN IN SWP BROTH AT DIFFERENT TEMPERATURES

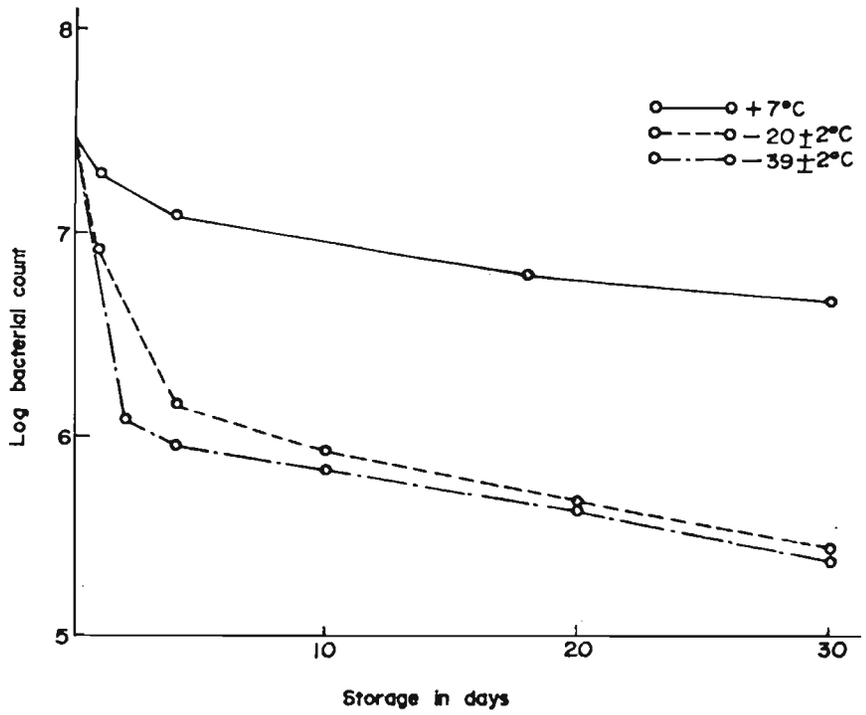

Fig.7.1.
SURVIVAL OF A VIBRIO STRAIN IN FISH MUSCLE MEDIUM AT DIFFERENT TEMPERATURES

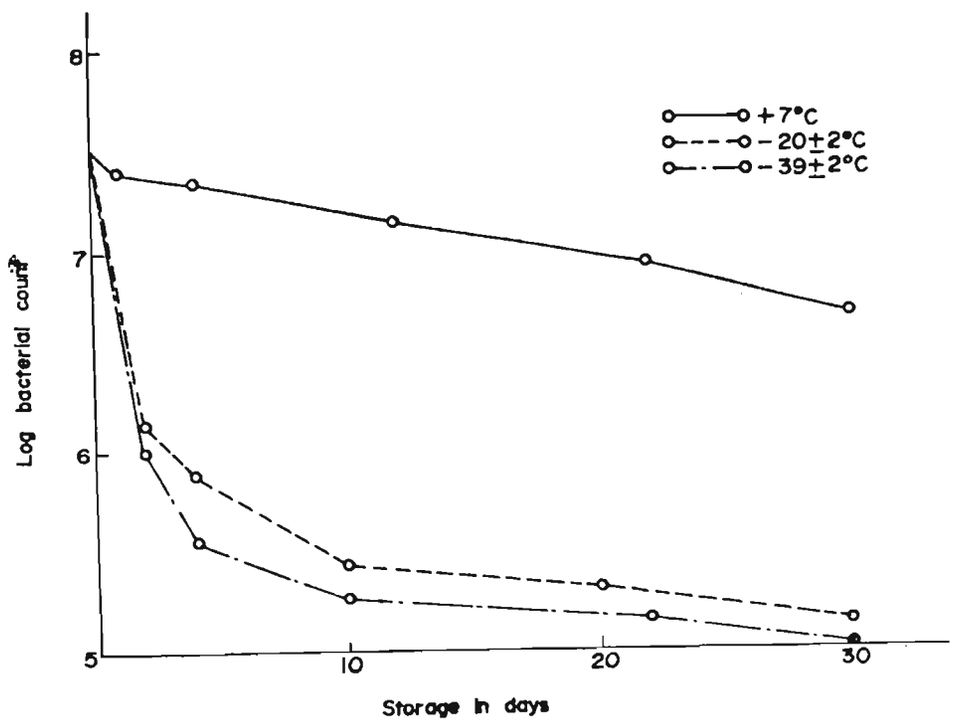

Fig.7.2.
SURVIVAL OF A VIBRIO STRAIN IN SWP BROTH AT DIFFERENT TEMPERATURES

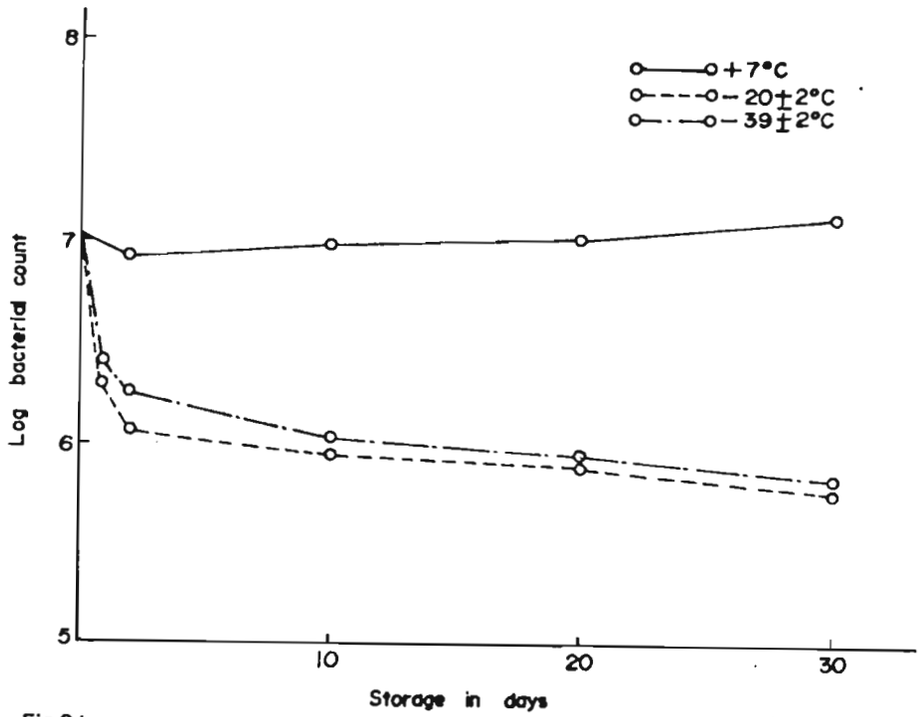

Fig.8.1.
SURVIVAL OF AN ACINETOBACTER STRAIN IN FISH MUSCLE MEDIUM AT DIFFERENT TEMPERATURES

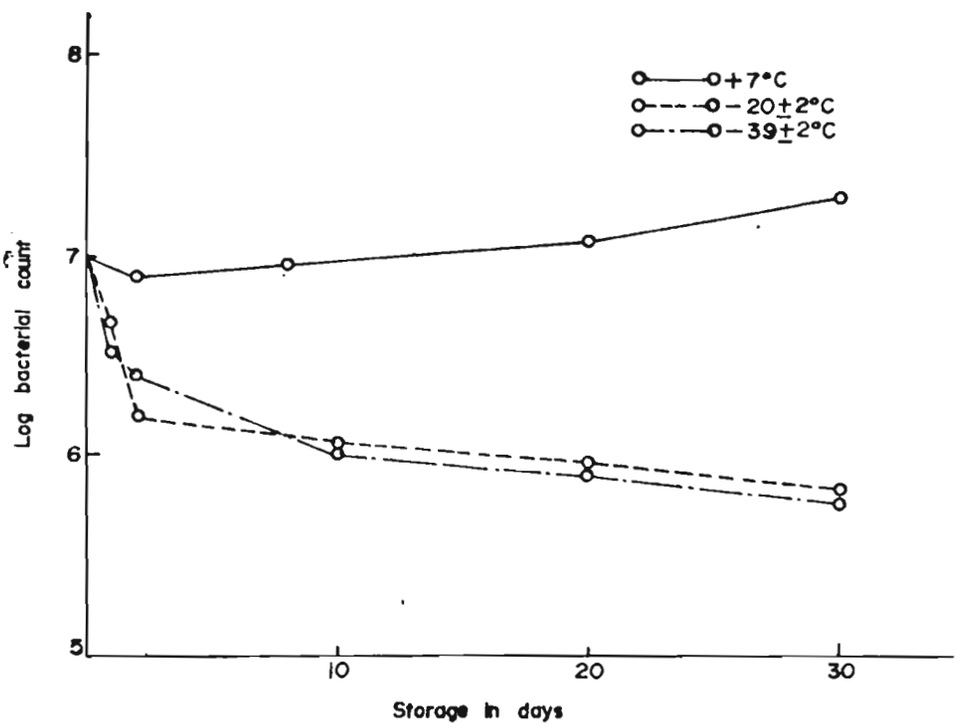

Fig.8.2.
SURVIVAL OF AN ACINETOBACTER STRAIN IN SWP BROTH AT DIFFERENT TEMPERATURES

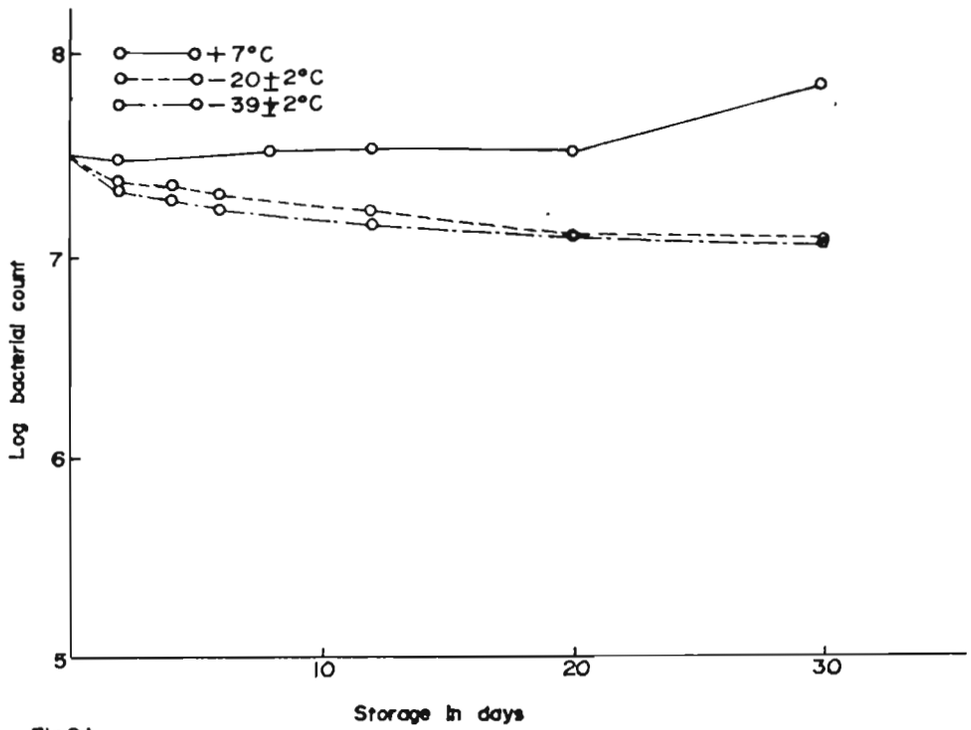

Fig.9.1.
SURVIVAL OF A MICROCOCCUS STRAIN IN FISH MUSCLE MEDIUM AT DIFFERENT TEMPERATURES

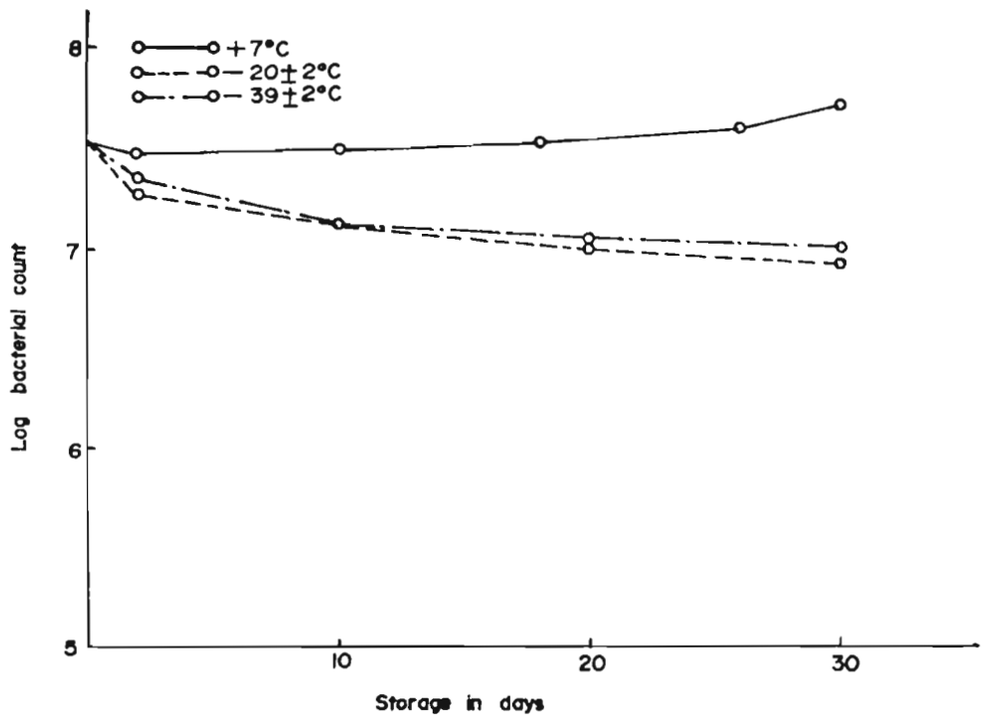

Fig.9.2
SURVIVAL OF A MICROCOCCUS STRAIN IN SWP BROTH AT DIFFERENT TEMPERATURES

To get a better idea of the mortality due to freezing and storage, the bacterial reduction was calculated as percentage of initial population after one day, 10 days and one month (Table 23), in fish muscle medium. This showed that most critical changes in cell number occurred in the early period of storage at -39±2°C. Also, the reduction occurring at the end of one month was the lowest in Micrococci and greatest in Vibrio species.

Since the overall reduction in the total initial population, illustrated by the above data is different for different bacterial species, it is possible that death rate during definite time intervals i.e. 0 - 24 hours, 24 h to 10 days and 10 days to 30 days could also be different. In this case the reduction in the population at given period with respect to the population just prior to that period was calculated. It was noted that in the case of Pseudomonas, 78.31% of the cells were destroyed in the first 24 hours, 54.45% of the remaining in 24 hours to 10 days and 24.3% of the remaining in the 10 to 30 days of storage at -39±2°C. This is quite in keeping with the observations from the graph also. Maximum destruction of viable cells occurred in the first 24 hours and the death rate decreased with subsequent storage period.

Table 23

Reduction of bacterial strains during freezing and frozen storage

	Percentage of reduction at -39±2°C						Percentage of reduction at -20±2°C					
	Storage (days)			Intervals of storage (days)			Storage (days)			Intervals of storage (days)		
	1	10	30	0-1	1-10	10-30	1	10	30	0-1	1-10	10-30
	1	2	3	4	5	6	7	8	9	10	11	12
2PW-5	78.31	90.12	92.53	78.31	54.45	24.30	65.06	88.07	92.64	65.06	65.86	38.28
SN-4	79.64	88.98	93.73	79.64	45.56	43.48	69.67	88.95	93.01	69.67	61.54	42.00
3LW-5	79.51	84.47	88.43	79.51	27.73	25.48	73.88	82.13	88.53	73.83	31.71	35.83
LS-1	12.69	31.76	45.07	12.69	21.85	18.05	14.63	31.48	39.72	14.63	19.74	12.03
SS-4	92.46	92.54	93.89	92.46	13.75	17.81	89.03	92.43	93.89	89.03	41.70	19.23
MB-398	67.30	83.34	97.51	67.30	64.34	70.10	49.05	94.45	96.59	49.05	89.10	38.69
PW-4	98.23	99.07	100.00	98.23	98.56	97.40	98.12	99.75	100.00	98.12	98.66	96.93
PD2T	97.02	99.96	99.99	97.02	98.53	73.40	96.80	99.87	99.98	96.80	95.90	87.13
PD-6	97.05	99.86	100.00	99.99	97.58	69.48	95.61	97.31	99.95	95.61	25.90	98.71
2LW-6	98.60	99.99	99.99	98.62	98.00	-	98.60	99.99	99.99	98.60	99.98	95.64
PD-4T	98.78	98.92	99.96	98.78	12.54	97.30	97.10	99.78	99.85	97.10	93.55	80.35
MB-407	76.02	97.68	99.97	76.02	90.34	98.86	73.47	97.52	99.74	73.47	6.06	90.38
LW-2	94.29	97.25	98.75	94.29	51.87	54.54	90.00	96.75	98.29	90.00	67.50	47.75
MS-8	68.89	97.69	98.34	68.89	92.57	41.27	69.07	97.57	98.25	69.07	92.39	27.95
2PW-2	42.29	72.40	81.89	42.29	52.17	66.93	31.09	62.93	90.65	31.09	46.21	74.78
PD-8	32.71	40.84	61.59	32.71	12.09	35.08	40.19	42.06	55.14	40.19	3.12	22.58

Table contd.

Table contd.

	1	2	3	4	5	6	7	8	9	10	11	12
SM-11	59.35	85.10	87.62	59.35	70.89	22.12	41.51	81.65	87.24	41.51	68.62	30.47
PW-24	70.10	75.28	79.04	70.10	15.05	11.17	65.42	74.10	79.94	65.42	25.08	19.69
LW-6	38.87	88.25	92.12	39.87	80.78	32.98	35.00	86.37	91.00	35.00	79.04	33.95
2PW-4	72.40	90.43	93.77	72.40	65.32	34.97	59.05	90.10	93.66	59.05	76.40	35.90
PW-9	63.64	89.50	94.75	63.64	71.13	50.00	57.37	87.64	91.80	57.37	71.00	33.67
SM-21	60.76	70.76	78.92	60.76	25.49	27.88	54.02	62.39	78.59	54.02	19.20	43.04
LM-3	63.71	87.79	91.96	63.71	66.34	34.15	49.29	86.99	91.60	49.29	74.33	35.46
MB-308	37.11	88.22	96.56	37.11	81.27	29.15	24.67	74.00	91.15	24.67	65.49	65.56
IMS-7	8.00	48.00	59.20	8.00	43.48	21.54	4.00	41.20	42.40	4.00	39.75	2.05
IMS-8	77.20	66.80	79.20	77.20	-	37.35	42.80	53.20	63.20	42.80	23.94	27.95
2MS-18	4.00	62.60	90.88	4.00	61.05	75.62	8.00	59.20	90.38	8.00	55.66	76.43
4MS-11	23.64	37.82	53.54	23.64	18.57	25.30	17.28	44.46	53.91	17.28	32.86	17.02
MB-244	42.86	77.62	90.69	42.86	70.84	13.72	38.10	75.95	79.83	38.10	61.15	16.14
4MG-8	24.05	39.78	44.29	24.05	19.40	9.01	19.04	29.46	41.88	19.04	12.87	17.61
MB-13	30.69	41.48	71.04	30.69	26.09	50.59	27.59	40.34	70.86	27.59	17.62	51.16
2LWP-2	23.79	31.03	66.20	23.79	4.77	51.00	17.24	47.24	66.90	17.24	36.25	37.25
CP-2	14.58	20.83	37.50	14.58	7.32	22.06	12.50	20.83	35.42	12.50	10.52	19.42
3PW-1	11.70	35.00	55.32	11.70	26.39	31.37	11.18	33.30	35.42	11.80	24.91	33.01
SM-14	27.88	56.95	84.72	27.88	40.39	64.52	26.39	56.95	85.42	26.39	41.51	66.13
PD-13	6.49	17.53	37.11	6.49	11.80	23.75	4.09	19.56	35.05	4.09	16.96	20.26
SM-10	70.83	89.34	94.67	70.83	63.43	50.00	33.64	70.00	84.34	33.64	52.63	47.78
LW-15	60.00	98.47	99.15	60.00	61.64	44.47	94.00	97.00	98.60	94.00	50.00	53.34
PW-21	94.84	98.77	99.12	94.84	76.13	29.38	92.84	98.70	99.07	92.84	84.09	28.21
7NG-3	90.62	92.47	95.57	90.62	19.79	42.10	88.93	90.82	94.23	88.93	16.23	37.08
8MG-4	35.65	43.57	69.32	35.65	12.31	43.86	26.74	41.56	58.42	26.74	20.28	29.81
6MX-7	80.83	84.58	87.92	80.83	19.57	21.63	79.38	82.71	87.29	79.38	16.17	27.51

Cultures 2PW-5 to MB-398 belong to Pseudomonas species; PW-4 to MB-407 are Vibrio species; LW-2 to PW-24 are Acinetobacter species; LW-6 to MB-308 Moraxella species; IMS-7 to 4MG-8 Flavobacterium species; MB-13 to PD-13 Micrococcus species and SM-10 to 6MX-7 Bacillus species

In the case of Moraxella, however, cultures 1 and 6 showed highest death during the period 24 hours to 10 days i.e. immediately after freezing. All the other four strains of Moraxella showed greater death of cells, ranging from 60.76% to 72.40% within the first 24 hours itself, showing more or less similar behaviour to the Pseudomonas strains.

Vibrio species were sensitive to freezing as well as frozen storage and this may probably account for their rather early disappearance from the frozen medium. At the same time, Micrococcus species were not very sensitive to the freezing period. But in all cases, the death increased gradually with storage period. In the case of Acinetobacter and Flavobacterium species a definite conclusion could not be drawn as the results were variable. However, four out of six strains showed sensitivity to freezing. For Bacillus also, maximum destruction was in the 0-24 hours period.

The effect of freezing and storage of bacterial cells at -20 ± 2°C was similar in all respects to the effect of freeing at -39 ± 2°C. But death was slightly of a lower level at -20 ± 2 compared to -39 ± 2°C in some strains, while the reverse occurred in some others. According to Ingram and Mackey (1976), lower the storage temperature and the less it fluctuate, the lower will be the rate of inactivation during storage. Various exceptions have been noted to this behaviour.

The highest temperature used for storage of bacterial cultures in this study namely, +7°C caused an increase in cell numbers during storage at that temperature, for all the cultures tested. The rise in cell number indicated growth and multiplication of the bacteria during that period. This shows that the selected temperature of +7°C falls above their minimum growth temperature. An exception was in the case of Vibrio strains for which there was gradual decrease in viable cells as indicated in the growth curve at that temperature (Fig. 7.1 and 7.2).

4.3.1.2 Survival of pathogenic/indicator bacteria at low temperature

Representative cultures of six strains of Salmonella including Salmonella anatum, Salmonella typhi, four cultures each of Escherichia coli, Staphylococcus aureus, Streptococcus fecalis and two cultures of Vibrio parahaemolyticus were studied in fish muscle media and nutrient broth (with 3% sodium chloride for strains of Vibrio parahaemolyticus). The temperatures used were +7°C, -29±2°C and -39±2°C.

The figures 10 to 14 represent changes occurring in the surviving population of selected pathogens over one year period. Survival curves, obtained in fish muscle media inoculated with Salmonella anatum and stored at -39±2°C

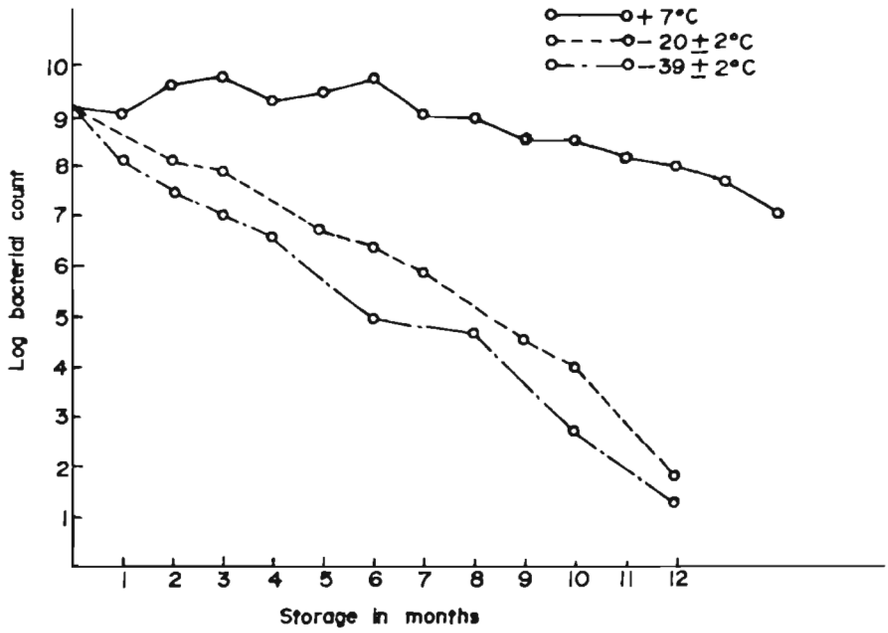

Fig.10.1.
SURVIVAL OF SALMONELLA ANATUM IN FISH MUSCLE MEDIUM AT DIFFERENT TEMPERATURES

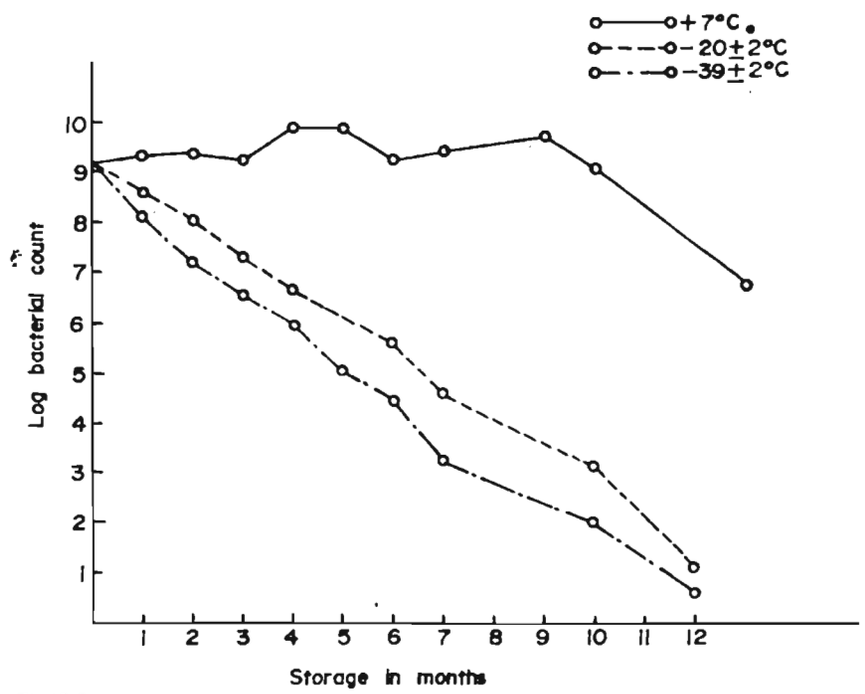

Fig.10.2.
SURVIVAL OF SALMONELLA ANATUM IN NUTRIENT BROTH AT DIFFERENT TEMPERATURES

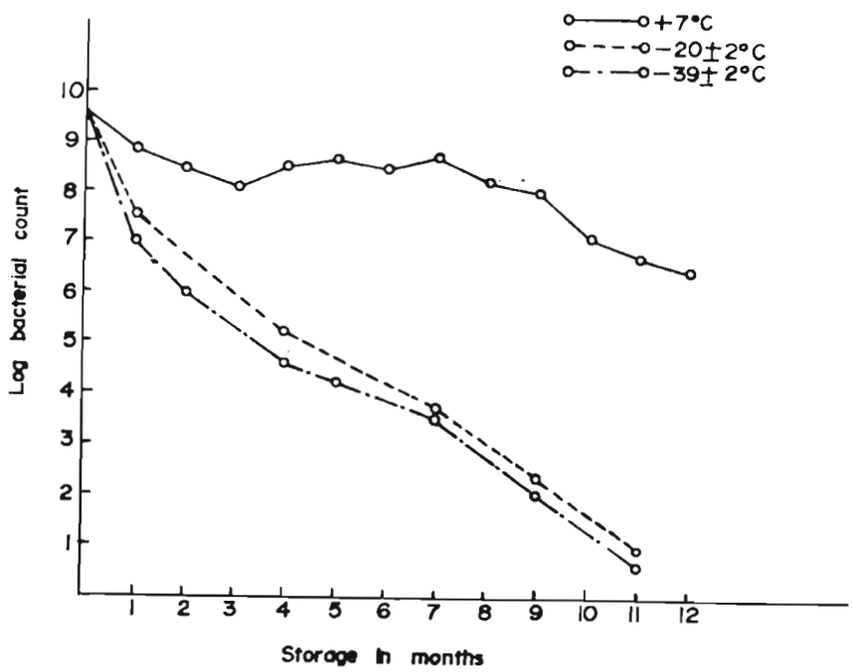

Fig.II.1
SURVIVAL OF ESCHERICHIA COLI IN FISH MUSCLE MEDIUM AT DIFFERENT TEMPERATURES

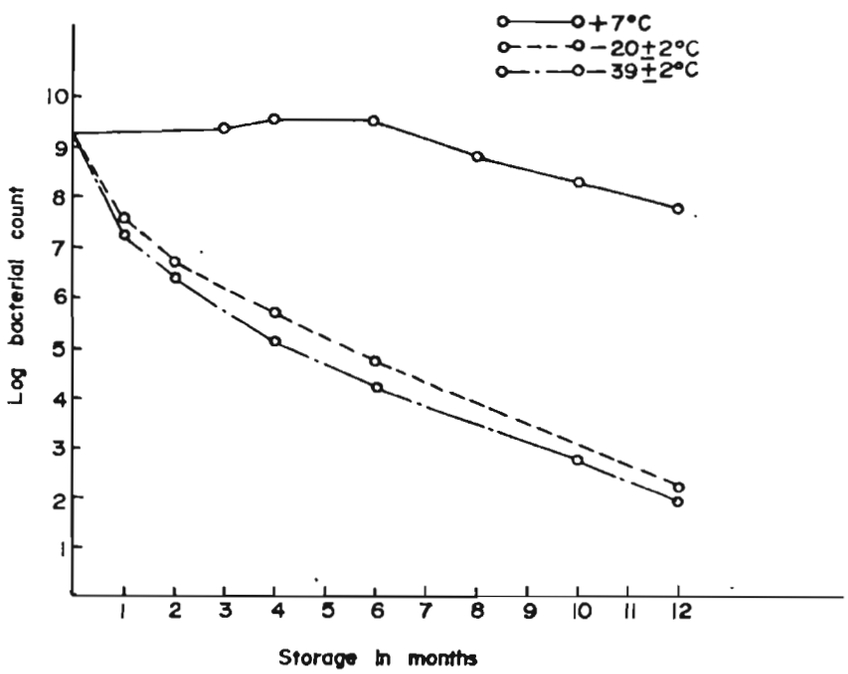

Fig.II.2.
SURVIVAL OF ESCHERICHIA COLI IN NUTRIENT BROTH AT DIFFERENT TEMPERATURES

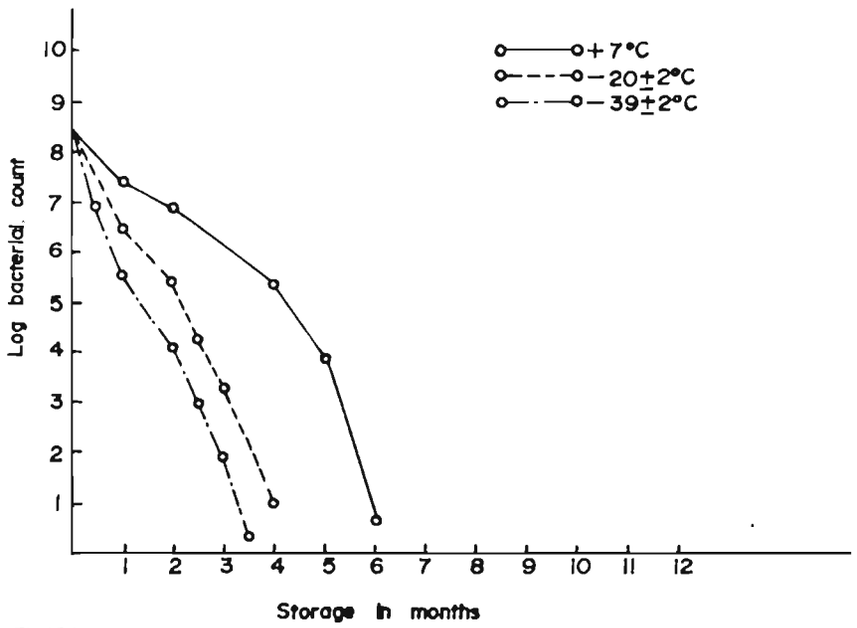

Fig.12.1.
SURVIVAL OF VIBRIO PARAHAEMOLYTICUS IN FISH MUSCLE MEDIUM AT DIFFERENT TEMPERATURES

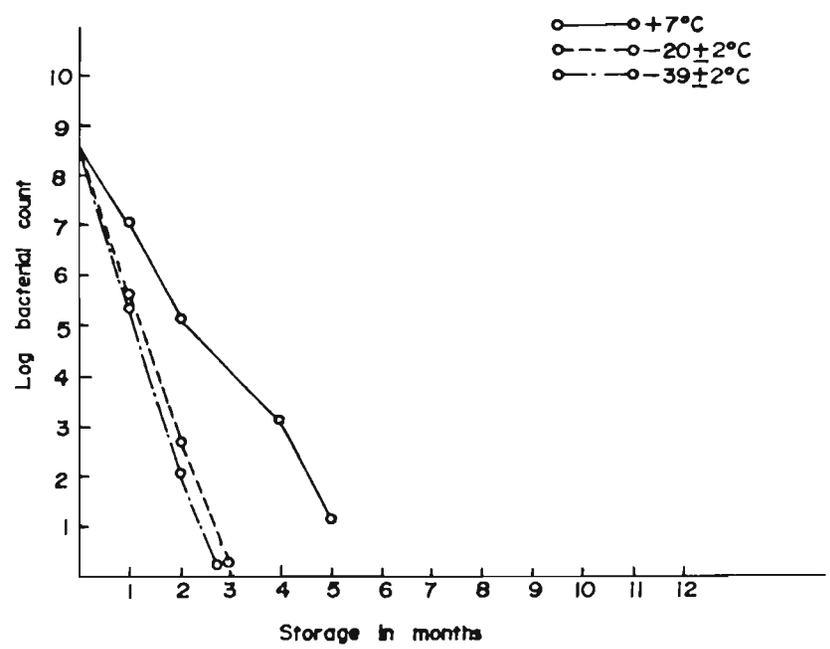

Fig.12.2.
SURVIVAL OF VIBRIO PARAHAEMOLYTICUS IN NUTRIENT BROTH AT DIFFERENT TEMPERATURES

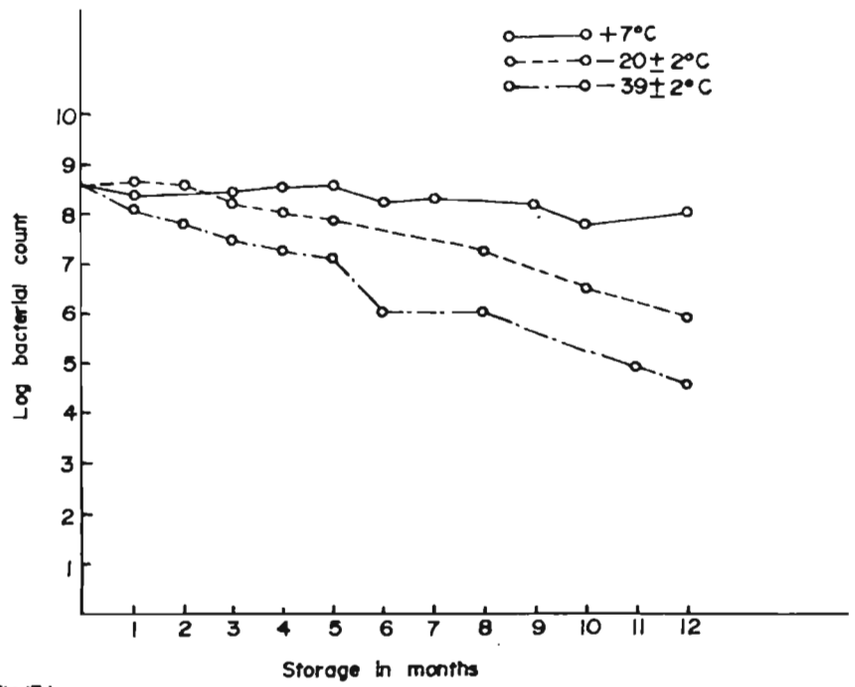

Fig.13.1
SURVIVAL OF A STAPHYLOCOCCUS STRAIN IN FISH MUSCLE MEDIUM AT DIFFERENT TEMPERATURES

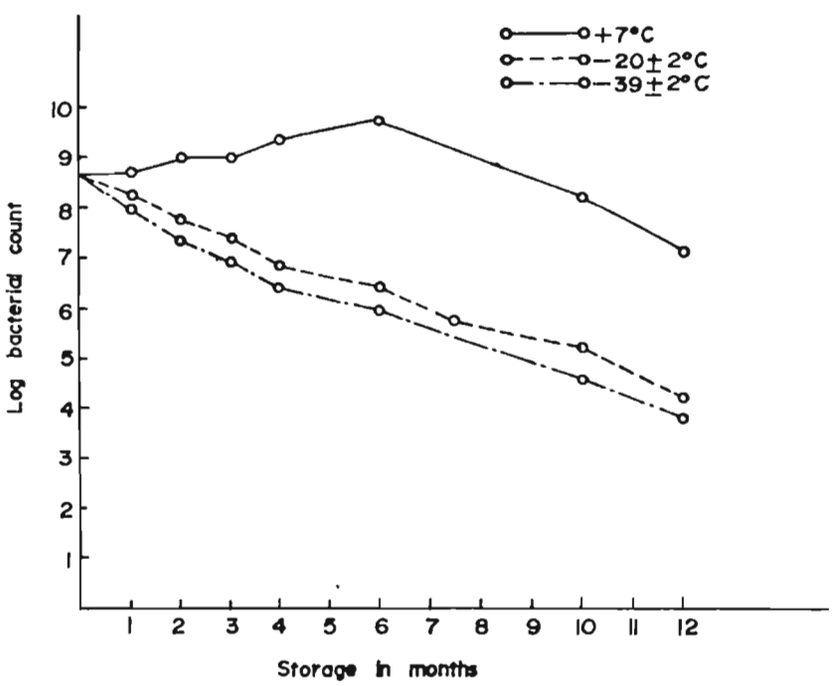

Fig.13.2.
SURVIVAL OF A STAPHYLOCOCCUS STRAIN IN NUTRIENT BROTH AT DIFFERENT TEMPERATURES

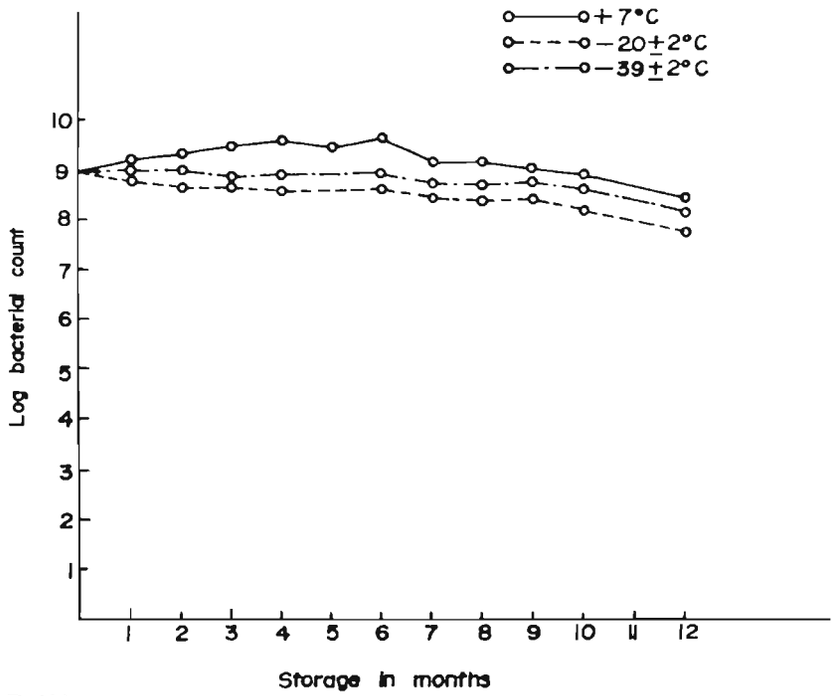

Fig.14.1
SURVIVAL OF STREPTOCOCCUS FECALIS IN FISH MUSCLE MEDIUM AT DIFFERENT TEMPERATURES

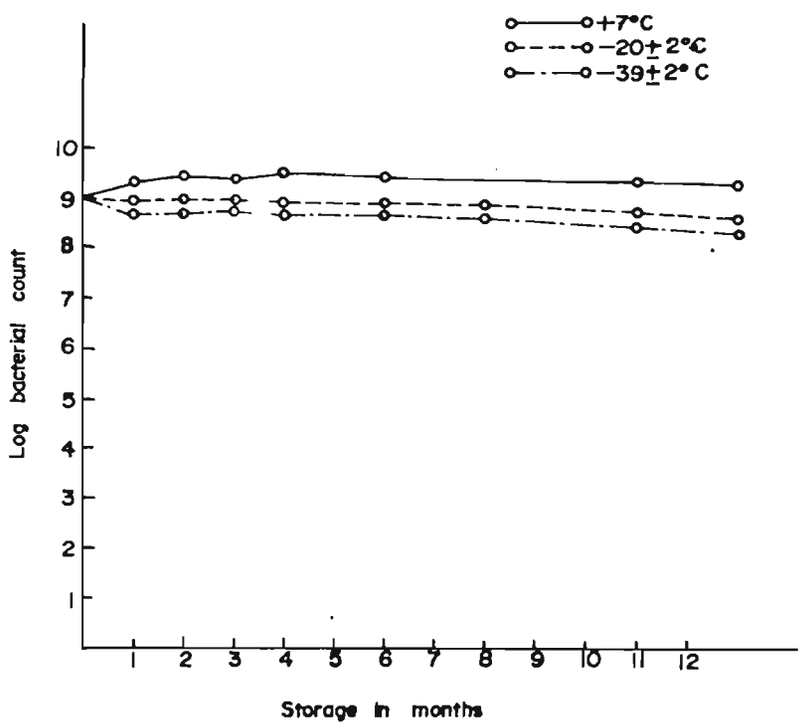

Fig.14.2.
SURVIVAL OF STREPTOCOCCUS FECALIS IN NUTRIENT BROTH AT DIFFERENT TEMPERATURES

showed rapid drop in the number of survivors from 1.07x 10^8/ml of the initial population to 5.01x10^4/ml after eight months. Behaviour of E. coli was more or less similar. But the survival of Staphylococcus aureus was slightly better than that of Salmonella species in fish muscle media.

In the case of Vibrio parahaemolyticus, there was a drastic reduction as indicated in the Figures 12.1 and 12.2. Eventhough a high cell number was used initially, there was total elimination of this species in the two media, at both temperatures.

At -39±2°C, the Streptococcus fecalis having an initial cell density of 10^9 cells/ml were reduced to 5.7x10^7 cells/ml at the end of one year period in fish muscle media.

It is seen that survival of Salmonella, Staphylococcus aureus and Vibrio parahaemolyticus were much higher in fish muscle media than nutrient broth. Exception was E. coli for which survival was found to be lower in fish muscle medium. In the case of Streptococcus fecalis, more or less Similar values were observed both in fish muscle media and nutrient broth.

Regarding the effect of temperature of freezing and storage, it was seen from the graph that for all the five

bacterial groups tested, survival was lower at the lower temperature viz. -39±2°C. But, a closer study of the data (Table 24), shows that the difference is negligible in most cases. For Salmonella typhimurium, the percentage reduction after 24 hours at -39±2°C and -20±2°C were 80.9 and 78.82 respectively. This type of behaviour was noted in two other strains also. One of the salmonella cultures isolated from fish even showed a higher survival at -39±2°C. This also points to the fact that the survival varies among the same species.

The rise in bacterial numbers at +7°C indicated that the minimum growth temperatures of all these strains except Vibrio parahaemolyticus lie below +7°C. A minimum temperature of growth of +10°C had been reported for Vibrio parahaemolyticus by Kaneko and Colwell (1973), +7°C by Vanderzant and Nickelson (1972) and +5°C by Beuchat (1973). A temperature of +5°C has been suggested as the minimum growth temperature of Staphylococcus (Keruluck et al., 1961) and Salmonella (Mossel et al., 1981) species.

Reduction as percentage of initial population of the bacterial cells and reduction occurring within fixed time intervals at -39±2°C and -20±2°C in fish muscle media are presented in Tables 24 and 25. This showed that for

Table 24

Reduction (%) of pathogenic/indicator bacteria during freezing and storage (as percentage of initial count)

	Temperature of freezing and storage					
	-39±2°C			-20±2°C		
	Storage (days)			Storage (days)		
	1	10	30	1	10	30
ST-3	80.94	91.15	97.75	78.82	90.11	96.10
SA-21	75.58	81.37	85.00	70.30	85.00	95.43
SC-135	83.23	88.67	94.28	84.10	87.92	94.20
CM-11	45.79	80.30	91.60	46.77	62.24	87.48
E-1	19.00	90.00	99.90	32.17	87.23	98.11
E-2	74.50	96.77	97.80	51.29	89.03	98.50
PN-38	41.93	99.06	99.30	40.92	98.12	99.07
SP-111	75.12	98.67	99.90	64.44	96.67	99.08
S-7	16.50	27.50	45.36	16.20	25.80	48.15
S-8	24.81	39.73	49.18	21.90	23.70	52.18
PR-12	20.98	46.15	65.93	13.18	42.85	62.63
PW-15	5.50	29.16	44.44	4.16	22.20	43.05
SF-2	19.00	20.00	45.34	12.50	22.50	30.00
SF-4	6.38	23.40	54.20	4.78	20.85	51.19
SC-9	7.71	33.76	67.50	12.54	35.69	51.76
SPM-1	5.50	14.03	24.20	7.50	11.67	21.13
VP-12	98.64	99.70	99.94	99.39	99.66	99.93
VP-11	99.87	99.99	99.99	98.79	99.17	99.99

Note: Cultures ST-3, SA-21, SC-135 and CM-11 belong to *Salmonella* species; E-1, E-2, PN-38, P-111 to *E. coli*; S-7, S-8, PR-12 and PW-15 to *Staphylococcus aureus*; SF-2, SF-4, SC-9 and SPM-1 to *Streptococcus fecalis* and VP-12 and VP-11 to *Vibrio parahaemolyticus*

Table 25

Reduction (%) during intervals of storage afer freezing of pathogenic/indicator bacteria

	Reduction (%) on storage at					
	-39±2°C			-20±2°C		
	Intervals of storage (days)			Intervals of storage (days)		
	0-1	1-10	10-30	0-1	1-10	10-30
ST-3	80.94	53.92	74.30	78.82	51.67	48.67
SA-21	75.58	40.47	46.80	70.30	48.60	35.17
SC-135	83.23	45.87	56.46	84.10	49.11	47.74
CM-11	45.79	63.79	57.67	46.77	53.80	62.41
E-1	39.03	74.30	87.40	32.17	72.71	82.48
E-2	74.50	87.34	53.00	51.29	81.60	86.76
PM-38	41.93	97.70	34.48	40.92	92.43	37.53
SP-111	75.12	94.68	76.09	64.44	85.67	73.91
S-7	16.50	11.96	24.50	16.20	11.49	25.60
S-8	24.81	9.87	22.67	21.90	2.73	29.92
PR-12	20.98	36.11	36.73	13.18	34.17	34.60
PW-15	5.50	25.00	21.56	4.16	23.18	22.60
SF-2	19.00	1.23	34.38	12.50	11.40	9.60
SF-4	6.38	18.18	40.27	4.78	16.87	39.20
SC-9	7.71	26.22	50.70	12.54	26.47	25.00
SPM-1	5.50	9.00	11.92	7.50	4.64	10.20
VP-12	98.64	78.18	80.40	98.39	79.23	81.85
VP-11	99.87	86.20	89.02	98.79	99.17	90.00

Note: Cultures ST-3, SA-21, SC-135 and CM-11 belong to *Salmonella* species; E-1, E-2, PM-38 and SP-111 to *E. coli*; S-7, S-8, PR-12 and PW-15 to *Staphylococcus aureus*; SF-2, SF-4, SC-9 and SPM-1 to *Streptococcus fecalis* and VP-12 and VP-11 to *Vibrioparahaemolyticus*

Salmonella anatum the maximum mortality was during freezing period i.e. within the 0-24 hours period. During frozen storage, the decline was more or less gradual and uniform. This was generally true for other strains of Salmonella also.

Among the four strains of E. coli, three showed maximum reduction in the count during the early period of frozen storage i.e. 24 hours to 10 days. Also, 97.9 to 99.9% of the initial population was reduced after storage for one month. This showed very high sensitivity of these strains to freezing temperatures.

The behaviour of Vibrio parahaemolyticus was very much identical with that of the Vibrio strains isolated from fish. The cultures were found to be sensitive to both freezing and frozen storage.

The Gram-positive cocci group, which consisted of Staphylococcus aureus and Streptococcus fecalis, were generally resistant to freezing and frozen storage. In this respect their behaviour was similar to that of the Micrococcus strains isolated from fish/shrimp. The ability of this Gram-positive group to grow at reduced water activity may be reason for their better survival in frozen substrates (Christian and Waltho, 1962).

On the basis of these results, the Vibrio

parahaemolyticus strains proved to be the most sensitive followed by Salmonella and E. coli strains. The Streptococcus fecalis strains were the most resistant and Staphylococcus aureus showed medium resistance to freezing and frozen storage. The study clearly shows that the survival at low temperature of pathogenic/indicator bacteria do not differ greatly from that of non-pathogens i.e. bacteria normally encountered in fish.

In the case of pathogenic bacteria, the survival pattern at low temperature has been studied widely. This may be mostly due to their clinical or hygienic importance. As such, very limited studies have been carried out on other types of bacteria. The early work of Hess (1934), on survival of marine bacteria, showed that freezing a cell suspension to -16°C resulted in 40-70% death in 8 minutes and 99.1% after 4-5 hours. Haines (1938) also made similar observation on Pseudomonas aeruginosa and E. coli, at -1°C and -5°C. Kiser and Beckwith (1942) noticed that for marine bacterial cultures, tentatively grouped as Achromobacter, storage at -28°C for 48 hours caused 92.4% death. Other reports in this field are those Arpai (1962) and Pogorzelska (1979). These reports are in good agreement with the data presented here.

In contrast, the fate of food poisoning (pathogenic) bacteria at temperatures below zero has been widely studied.

(Gunderson and Rose, 1948; Raj and Liston, 1961; Georgala and Hurst, 1963; Shiflett et al., 1967; Digirolamo et al., 1970; Matches et al., 1971; Covert and Woodburn, 1972; Zawadzki and Pogorzelska, 1975; Hall and Slade, 1980.

The results presented in this study are supported by the following works. Raj and Liston (1961) studied the survival of major groups of pathogenic bacteria in seafoods at freezing temperatures. They observed somewhat irregular decline in bacterial count of Salmonella in first 130 days and a 10 fold decrease over 393 days. For Salmonella species, in minced meat, there was 10.5 to 15.2% reduction after freezing, 49.5 to 54.7% after storage at -23°C for 10 days and 88.1 to 99.2% after 10 months (Zawadzki and Pogorzelska, 1975).

In frozen suspensions staphylococci are themselves more resistant to cold storage than Gram-negative rods such as Salmonella (Georgala and Hurst, 1963). Staphylococcus aureus showed 7-fold decrease in 130 days of storage at 0°C after freezing in fish muscle (Raj and Liston, 1961).

Vibrio parahaemolyticus showed high sensitivity to refrigeration and freezing temperatures. Vanderzant and Nickelson (1972) found that viable count of Vibrio parahaemolyticus in whole shrimp, stored at 3°C, 7°C, 10°C

and -18°C, showed sharp decline in the first 8 days. After 8 days at -18°C, an initial cell suspension of 10^5 cells/g of shrimp was reduced to 10^3 cells/g.

Enterococci were more resistant to freezing and frozen storage than Staphylococcus or other Gram-positive rods. (Dussault, 1956; Raj and Liston, 1961; Lakshmy, 1964; Digiloramo et al., 1970) and same trend was found here.

Tanner and Williamson (1928) stated that during storage of bacteria at low temperature "rate of death follows the curve of monomolecular reactions, the death being proportional to number of viable cells". Hence the plot of logarithm of the count of survivors against period of storage should result in a straight line. The survival curves obtained in this study was not linear. Similar curves were also reported by Haines (1938), Harrison et al. (1953) and Major et al. (1954) for cultures like E. coli, Pseudomonas aeruginosa and Aerobacter aerogenes. The deviation from the straight line curve may be due to differing death rate of microorganism at freezing temperatures. The death rate of microorganism has been shown to depend mainly on the bacterial type and chemical composition of the product (Kuzminska, 1982).

Many of the early reports show that low freezing temperatures near -20°C are less harmful than the medium

range of temperature such as -10°C (Wieser and Osterud, 1945; Borgstrom, 1955; Straka and Stokes, 1959). This holds true in large number of studies conducted mainly on pathogenic bacteria.

Hartsell (1951) observed better survival of Salmonella in frozen egg at -17°C than -1°C. Similarly, Georgala and Hurst (1963) noted that on storage of Salmonella typhimurium in comminuted beef for 3 months, 50% of the cells survived at -20°C as against 1% at -2°C. Digirolamo et al. (1970) noted for Salmonella derby and E. coli a higher survival at -30°F than +32°F.

But, a close scrutiny of the data shows that there are also reports in which a higher mortality was observed at lower temperature (Kiser and Beckwith, 1942; Ray et al., 1969 ; Kuzminska, 1982). But such reports mostly pertain to temperature below -20°C.

The influence of the freezing menstruum may also be an important factor. Covert and Woodburn (1972) noticed that addition of 3% sodium chloride to fish muscle media improved the count at -18±1°C with result that survival was more at -18±1°C than -5±1°C. Asakawa (1967) noted greater decline of Vibrio parahaemolyticus in laboratory media at -10°C than -20°C, while in raw tuna meat survival was same at both -20°C and -10°C.

4.3.2 Behaviour of bacteria in mixed population during freezing and frozen storage

4.3.2.1 *Pseudomonas* and *Moraxella* strains

The bacteria used in the study consisted of a *Pseudomonas* strain (*Pseudomonas* 2PW-5) and a *Moraxella* strain (*Moraxella* 2PW-4).

Selection of the culture:

The behaviour of the two types of bacteria towards 2.5 IU of penicillin (Benzyl penicillin, 5,00,000 units) was made use of in this study. While 98% of the *Pseudomonas* species were insensitive to 2.5 IU of Penicillin , 99% of *Moraxella* strains proved to be sensitive to such a concentration of penicillin. The sensitive cultures used in the study exhibited an inhibition zone ranging from 0.8 mm to 1.2 mm diameter. Three cultures of *Moraxella*, showing highest sensitivity, were chosen for further screening. Three *Pseudomonas* were randomly selected. The inhibition zones were developed by placing filter paper (Whatman No.40) discs of 0.5 mm carrying 2.5 IU penicillin over plates seeded with 18 hour culture of the bacteria grown in SWP at RT.

Sreening of the cultures:

The recovery of *Pseudomonas* and *Moraxella* strains at room temperature on antibiotic agar (ABA) and antibiotic

agar supplemented with 3 µg/ml of Pencillin (ABAP) was studied. Eventhough 2.5 IU of penicillin was used for primary selection, for further studies a concentration of 3 ug of penicillin per ml of agar medium was used. This was to ensure the complete suppression of Moraxella sp. in ABAP subsequently. These cultures were frozen at -20±2°C for 2 weeks and again subjected to this screening (Table 26). The results showed that the recovery for the fresh and frozen cells of Pseudomonas were almost equal in ABA and ABAP while that of Moraxella were negligible in ABAP. From the three strains, a Pseudomonas strain (2PW-5) and Moraxella (2PW-4) were finally chosen.

As a means of cross checking the method, the mixture of known cell concentrations were prepared from the Pseudomonas and Moraxella strains. The count of the individual strains were estimated by the penicillin method and also by plating on TSA (Table 27).

The behaviour of the mixed population of Pseudomonas strain and Moraxella strain on freezing at -39±2°C and storage in fish muscle is given (Table 29). The survival percentage of the Pseudomonas and Moraxella in mixture were 34.6 and 49.9 after 24 hours, 10.9 and 28.59 after 1 month, 0.71 and 1.97 after 4 months and 0.1 and 1.04 after 7 months of storage. The Pseudomonas, when frozen

Table 26

Recovery of Pseudomonas and Moraxella strains on ABA and ABAP (Bacterial count/ml)

		Count* on ABA	Count* on ABAP
Pseudomonas	IMS-27 (fresh)	1.07×10^7	9.9×10^6
"	" (frozen)	6.18×10^6	6.01×10^6
"	2PW-5 (fresh)	1.16×10^8	1.02×10^8
"	" (frozen)	7.30×10^7	7.20×10^7
"	3MS-5 (fresh)	3.50×10^7	3.30×10^7
	" (frozen)	9.20×10^6	9.20×10^6
Moraxella	IMS-3 (fresh)	1.49×10^3	10^2
"	(frozen)	8.82×10^7	Nil
"	2PW-4 (fresh)	4.70×10^7	10^2
"	" (frozen)	9.98×10^6	10^2
"	PW-9 (fresh)	8.66×10^7	Nil
"	" (frozen)	3.58×10^7	10^2

*Bacterial count taken at RT (29±2°C)

Table 27

Comparison of the data obtained using penicillin method with that of plating on TSA (Bacterial count/ml)

Experiment No.*	Count on ABA	Count on ABAP	Count on ABA - count on ABAP	TPC on TSA: Pseudomonas	TPC on TSA: Moraxella
1	1.75×10^8	1.37×10^8	3.8×10^7	1.34×10^8	3.5×10^7
2	4.5×10^8	2.2×10^8	2.3×10^8	2.1×10^8	2.1×10^8
3	1.4×10^8	2.4×10^6	1.16×10^8	2.3×10^8	1.39×10^8

*Bacterial count taken at RT (29±2°C). Only representative values are given.

individually at this temperature showed a survival of 32.7% after 24 hours, 12.84 after 1 month and 0.33% after 4 months. The corresponding figures for Moraxella were 40.24%, 22.1% and 0.8%.

This showed that for strains studied here, the survival of Pseudomonas during frozen storage in mixed culture was almost similar to that of individually frozen culture. This was not the case with Moraxella strain. It showed less sensitivity, when present in a mixture, to freezing temperature.

4.3.2.2 Pseudomonas and Micrococcus strains

The ability of the Pseudomonas strain to produce acid from sucrose was noticed in many strains, while majority of Micrococcus spp. failed to do so. This difference was made use of for enumerating Pseudomonas in presence of Micrococcus strain.

The peptone sucrose agar (PSA) was developed for this purpose. The recovery in this media compared well with the recovery on TSA, for fresh as well as frozen cells of Pseudomonas and Micrococcus (Table 28). The Pseudomonas cells attacking sucrose turned yellow, whereas the Micrococcus remained colourless.

The survival of the pure cultures of Pseudomonas and

Table 28

Recovery of Pseudomonas sp. and Micrococcus on PSA media before and after freezing (Bacterial count/ml)

	Trial No.	*Count on PSA	*Count on TSA
Pseudomonas sp. (unfrozen cells)	1	9.38×10^7	1.19×10^8
	2	2.1×10^7	2.88×10^7
	3	3.7×10^7	3.9×10^7
Micrococcus sp. (unfrozen cells)	1	1.7×10^7	1.9×10^7
	2	2.08×10^8	1.98×10^8
	3	4.11×10^7	4.83×10^7
Pseudomonas frozen for 1 week at $-39 \pm 2°C$	1	3.21×10^7	4.87×10^7
	2	7.13×10^6	8.34×10^6
	3	9.89×10^6	2.11×10^7
Micrococcus frozen for 1 week at $-39 \pm 2°C$	1	2.01×10^8	3.11×10^8
	2	5.09×10^7	5.04×10^7
	3	3.43×10^7	3.67×10^7

*Bacterial count taken at RT ($29 \pm 2°C$). Only representative values are given

Table 29

Changes in the survival of a *Pseudomonas* and *Moraxella* strains when frozen individually and together at -39±2°C in fish muscle

Period	Percentage of *Moraxella* ++	Percentage survival of *Pseudomonas*	Percentage survival in the mixture of *Moraxella*	Percentage survival in the mixture of *Pseudomonas*
24 h	40.24*	32.70	49.90	34.60
1 month	22.10	12.84	28.59	10.90
4 months	0.80	0.33	1.97	0.71
7 months	0.10	0.10	1.04	0.10

*Bacterial count taken at RT (29±2°C)

++As percentage of initial population

Table 30

Changes in the survival of the *Pseudomonas* and *Micrococcus* strains frozen individually and together at -39±1°C in fish muscle

Storage period	Percentage survival of *Pseudomonas* strain ++	Percentage survival of *Micrococcus* strain	Percentage of survival in mixture of *Pseudomonas*	Percentage of survival in mixture of *Micrococcus*
24 h	32.0*	70.3	37.0	68
1 month	13.2	30.9	14.8	34
4 months	1.12	28.0	0.09	26
7 months	0.2	20.0	0.01	18

*Bacterial count taken at RT (29±2°C)

++As percentage of initial count (0 h)

Micrococcus and their mixture when frozen and stored at -39±2°C in fish muscle are given in Table 30.

The survival of the Pseudomonas strain after 24 h, 1 month and 7 months were respectively 32%, 13.2% and 0.2%. The survival of the Micrococcus species after the corresponding periods of freezing and storage were 70.3%, 30.9% and 20% respectively. When these two were mixed together and frozen, the survival of Pseudomonas was found to be 37%, 14.8% and 0.01% after 24 hours, 1 month and 7 months. The Micrococcus strain in the mixture survived 68%, 34% and 18% during these periods.

These results indicate that the survival of the Gram-negative Pseudomonas and Gram-positive Micrococcus were almost similar, when frozen individually or in mixture.

4.3.2.3 Pigmented and nonpigmented Gram-positive cocci

The mediumselected for this study was skim milk agar, which was nutrient agar supplemented with 10% solution of skim milk. The pigment production was intense in this mediumand colonies of pigmented strain were readily visible.

When incubated at room temperature, the bacterial count of pigmented cocci decreased from 1.585×10^7 cells/ml to 6.36×10^6 cells/ml as a result of freezing at -39±2°C

for 24 hours. This corresponded to a survival of 39.75%. The survival after 1 month, 4 months and 7 months of this strain were respectively 19.94%, 7.94% and 2.88% (Fig.15).

The bacterial count of the non-pigmented cocci at room temperature decreased from 9.12×10^7 cells/ml to 1.25×10^7 cells/ml showing a survival of 13.7% after 24 hours at $-39 \pm 2°C$. After 1, 4 and 7 months of storage, the survival of this organism decreased to 8.7%, 1.58% and 0.43% respectively. This showed that non-pigmented strain was more susceptible to temperature of $-39 \pm 2°C$. This observation is supported by the work of Grinsted and Lacey (1973), who showed that on storage pigmented varieties of Staphylococcus produced non-pigmented varieties which were more susceptible to dessication and linoleic acid than the corresponding wild strains.

Eliott and Michener(1960) stated that during frozen storage the viability of mixture of microorganisms remained constant in natural foods. While the behaviour of Pseudomonas and Micrococcus species were same in the mixture as well as individual cell suspensions, the behaviour of Moraxella was slightly different. An important factor in the survival of bacteria in unfavourable circumstances is the ability of the cells

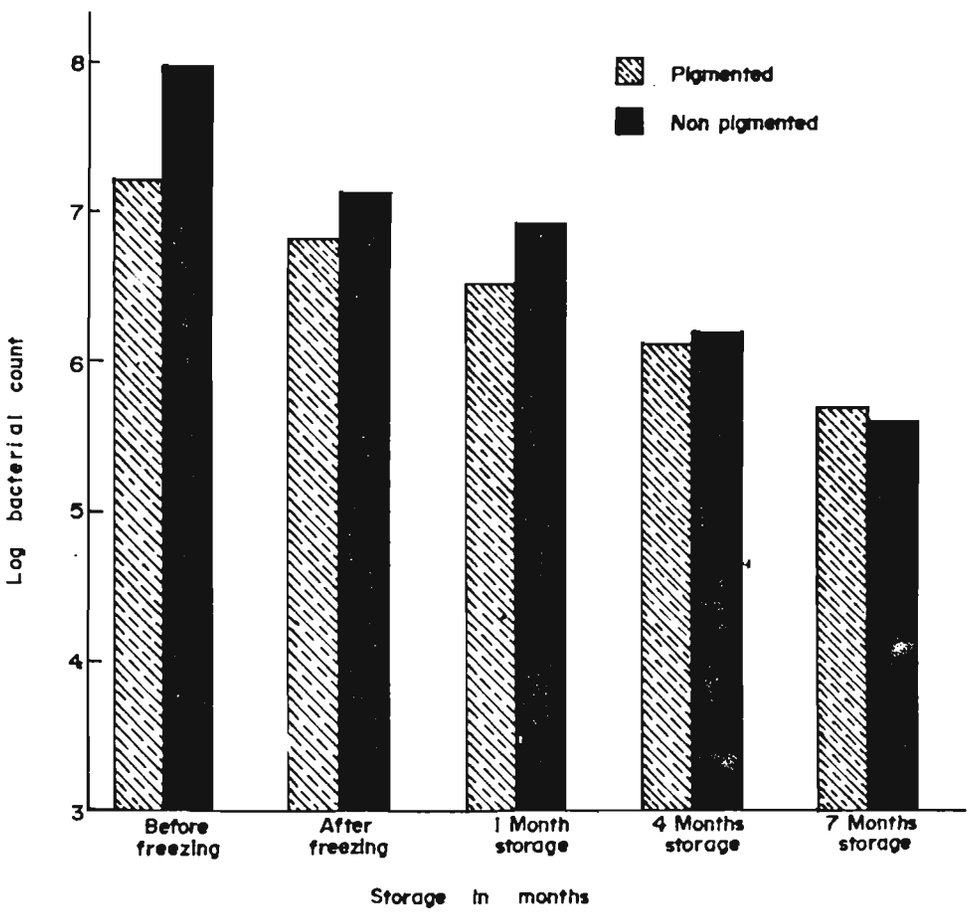

Fig.15.
CHANGES IN THE SURVIVAL OF A PIGMENTED AND A NON-PIGMENTED GRAM +VE COCCI WHEN FROZEN TOGETHER AND STORED AT $-20 \pm 2°C$.

to utilize substances made available by degradation of various endogenous macromolecular constituents (Strange et al., 1961). Hence, in a mixture of two bacterial species the species having a better ability to use the constituent may survive better and this may be the reason for the better survival of Moraxella in comparison to Pseudomonas sp.

Eventhough voluminous data has accumulated regarding the growth of mixture of microorganisms during thawing, such data on the survival of microorganisms during freezing is very scanty. Hence, this work is of importance.

4.3.3 Factors influencing survival during freezing and frozen storage

4.3.3.1 The effect of freezing menstruum on the survival of bacteria during freezing and frozen storage

The survival of selected bacterial cultures in different freezing menstrua, after freezing and storage at $-20\pm2^{\circ}C$, are illustrated in figures 16.1 to 16.8.

Three cultures each of the Pseudomonas, Moraxella, Acinetobacter, Flavobacterium/cytophaga, Micrococcus and Bacillus were studied. Typical data is presented.

For Pseudomonas spp., maximum survival after 24 hours was noted in fish muscle media and minimum in distilled water. The percentage of surviving population

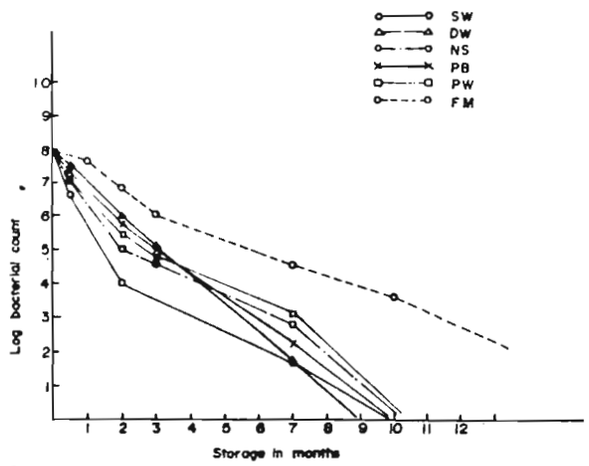

Fig. 16.1.
SURVIVAL OF A PSEUDOMONAS STRAIN IN VARIOUS SUSPENDING FLUIDS DURING FREEZING AND STORAGE AT -20±2°C

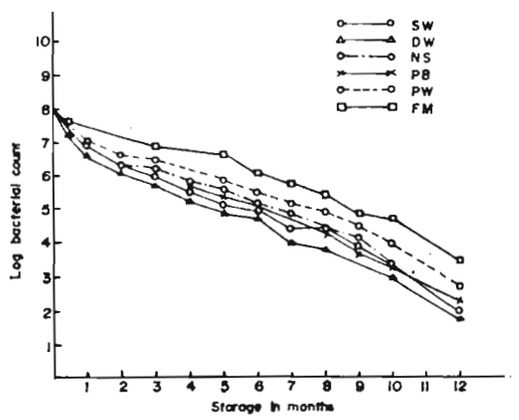

Fig. 16.2.
SURVIVAL OF A MORAXELLA STRAIN IN VARIOUS SUSPENDING FLUIDS DU
FREEZING AND STORAGE AT -20±2°C

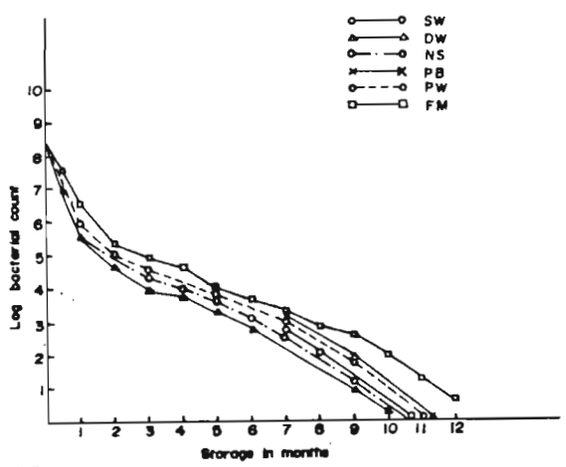

Fig. 16.3.
SURVIVAL OF AN ACINETOBACTER STRAIN IN VARIOUS SUSPENDING FLUIDS DURING FREEZING AND STORAGE AT -20±2°C

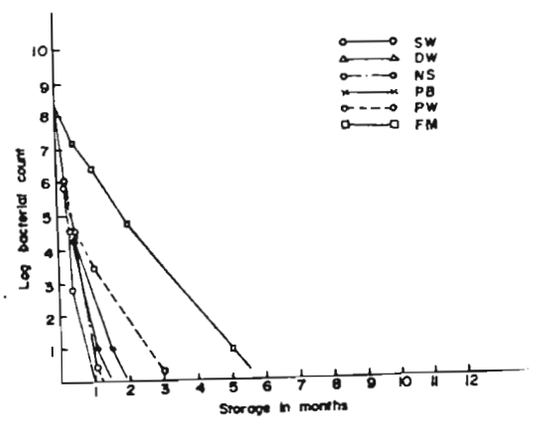

Fig. 16.4.
SURVIVAL OF A VIBRIO STRAIN IN VARIOUS SUSPENDING FLUIDS DU
FREEZING AND STORAGE AT -20±2°C

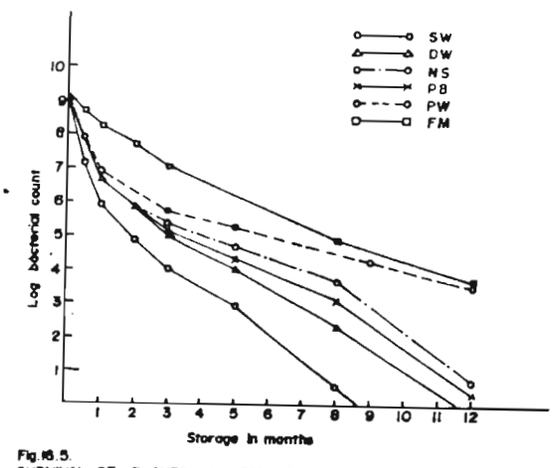

Fig.16.5.
SURVIVAL OF ESCHERICHIA COLI IN VARIOUS SUSPENDING FLUIDS DURING FREEZING AND STORAGE AT -20±2°C

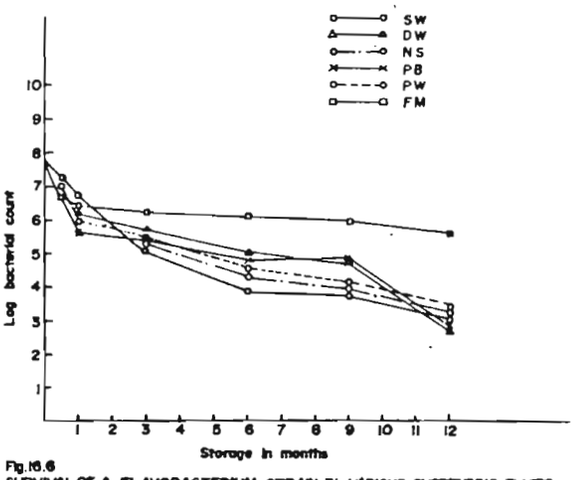

Fig.16.6
SURVIVAL OF A FLAVOBACTERIUM STRAIN IN VARIOUS SUSPENDING FLUIDS DURING FREEZING AND STORAGE AT -20±2°C

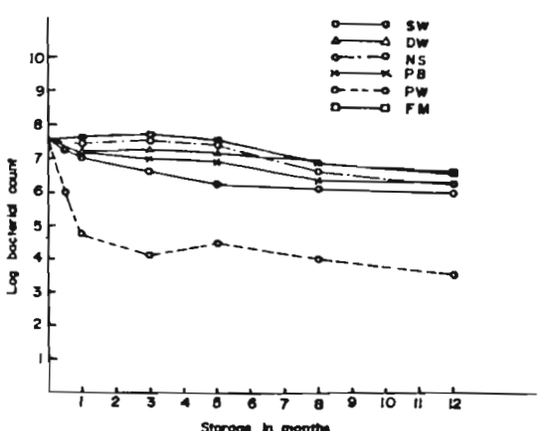

Fig.16.7
SURVIVAL OF A MICROCOCCUS STRAIN IN VARIOUS SUSPENDING FLUIDS DURING FREEZING AND STORAGE AT -20±2°C

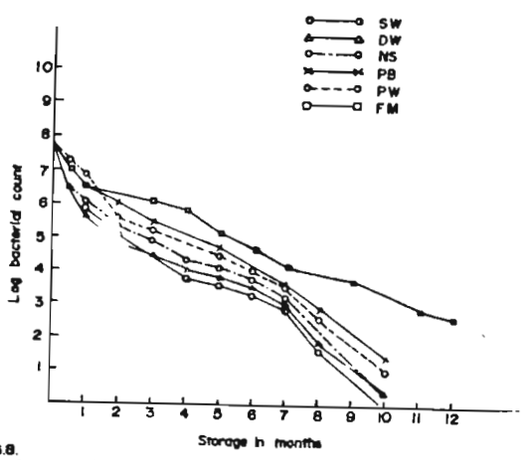

Fig.16.8.
SURVIVAL OF A BACILLUS STRAIN IN VARIOUS SUSPENDING FLUIDS DURING FREEZING AND STORAGE AT -20±2°C

in sea water, distilled water, n.saline, phosphate buffer, peptone water and fish muscle media were respectively 33, 12, 0.37, 0.37, 33 and 42 respectively. But,on storage, the trend was found to change. With an initial cell density of 7.3×10^7 cells/ml, the cells, surviving after 6 months in sea water and distilled water were 10^2 cells/ml. While in n.saline 600 cells/ml survived, in peptone water 1.4×10^3 cells/ml survived after freezing and 8.56×10^4 cells/ml in fish muscle media. This showed that fish muscle media is the most protective during freezing as well as on storage for Pseudomonas sp. This was found to be true for other cultures as well. It is seen from the figure that the difference in the survival of Pseudomonas in different fluids were most marked in the early period of storage. By about eight months, all fluids except fish muscle media proved to be lethal to the same level. This type of behaviour was noted for other cultures namely, Moraxella, Acinetobacter, Flavobacterium and Micrococcus species.

The freezing menstruum,for which lowest survival existed, differed for the bacterial cultures. Sea water proved to be the least protective for cultures of Bacillus, Flavobacterium, E. coli, Vibrio and Pseudomonas. However, a decrease in the survival of Moraxella and Acinetobacter occurred in distilled water. For Micrococcus,

the survival was lowest in peptone water. The protective effect of other fluids were intermediary.

It is also evident from the figures that just as the survival rate of these cultures varied from fluid to fluid, the surviving period of a given culture also varied. While Pseudomonas cells survived in sufficient numbers in fish muscle for one year, their survival in distilled water did not exceed 8 months. In sea water, phosphate buffer and normal saline these cells survived up to 9 months. This effect was clearly visible in the case of Vibrio spp., where cells remained viable up to 5 months in fish muscle media and to less than 3 months in other fluids.

This data evidently points to the protective effect afforded by fish muscle and its superiority over others, as the most protective environment. According to Raj and Liston (1961), the protection afforded by the fish muscle substrate may be due to the fish protein molecule, which behave as a true colloid and interfere with the rate of crystallisation of water in the fish. By minimising the loss of water from the cell, they prevent the denaturation of proteins within the cells.

According to the present study, sea water was the least protective environment for most of the bacterial cultures. Reports of their survival in sea water is

rather scanty. But considerable death of the bacteria, Aerobacter aerogenes, in distilled water was reported by Postgate and Hunter (1961). According to them, the possibility of the presence of dissolved impurities which accounted for the kill was negligible since they obtained comparable deaths in distilled water purified by resin treatment and double distillation with that of ordinary distilled water. Contrary to the finding of Postgate and Hunter (1961), Clement (1961) found distilled water to be rather harmless environment for freezing of A. aerogenes. They attributed this to the species difference. Two of the cultures tested here viz. Moraxella and Acinetobacter sp. exhibited maximum death in distilled water.

There are several reports on the toxicity of saline on bacteria during freezing. According to Nakamura and Dawson (1962), saline was more toxic environment than nutrient broth for Pseudomonas fluorescens during freezing and frozen storage. Toxicity of saline was also noticed by Major et al. (1955) and Harrison (1956). Calcott and MacLeod (1974) reported saline to be more toxic environment than distilled water for E. coli. But according to the present investigation, for E. coli cells, saline was more toxic than fish muscle and peptone water. But it was less

toxic than distilled water. Exception was *Flavobacterium* species for which saline was more toxic than distilled water.

Calcott and MacLeod (1974) also noted that salts other than sodium chloride, such as lithium chloride, potassium chloride, sodium sulphate and potassium sulphate, were equally lethal as freezing menstrua. The severe toxicity of sea water may be due to the high salt concentration.

4.3.3.2 Effect of initial cell number

The influence of the initial population on the survival of selected strains of bacteria during storage at low temperatures were studied in fish muscle media, at two temperatures viz. -20±2°C and -39±2°C. The results are presented in Tables 31 and 32.

The theory of population effect, put forward by Major *et al.* (1955), necessitates that the relative drop in viable count at anytime during frozen storage should be proportional to the initial count. In other words, the fraction of the cells that survives at a given period is proportional to the initial cell number. If C_1 represents the initial cell population and C_2 that after time 't',

$$\frac{C_2}{C_1} \propto C_1 \text{ or } C_2 = k(C_1)^2 \text{ or } K = \frac{C_2}{(C_1)^2}$$

where K is a constant.

Table 31

Influence of initial cell population on the survival of bacterial strains in fish muscle media during freezing and storage at -20±2°C for one month

Bacterial strain	Concentration of cells/ml			
	Initial cell number	Cell number ratio after one month		$K = \frac{(C_2)}{(C_1)^2}$
	C_1	C_2	$\frac{C_2}{C_1}$	
Escherichia coli	2.1×10^{8}	1.78×10^{8}	8.5×10^{-1}	4.03×10^{-7}
	2.1×10^{7}	3.2×10^{6}	1.5×10^{-1}	7.2×10^{-7}
	2.1×10^{6}	4.4×10^{4}	2.0×10^{-2}	9.97×10^{-7}
	2.1×10^{5}	2.9×10^{3}	1.3×10^{-2}	6.6×10^{-6}
Pseudomonas sp.	3.7×10^{8}	1.2×10^{8}	3.2×10^{-1}	8.8×10^{-10}
	3.7×10^{7}	8.4×10^{6}	2.2×10^{-1}	6.13×10^{-9}
	3.7×10^{6}	6.2×10^{5}	1.6×10^{-1}	4.5×10^{-8}
	3.7×10^{5}	2.4×10^{4}	6.4×10^{-2}	1.75×10^{-7}
Micrococcus sp.	3.2×10^{7}	1.1×10^{7}	3×10^{-1}	1×10^{-8}
	3.2×10^{6}	1.8×10^{6}	5×10^{-1}	1.7×10^{-7}
	3.2×10^{5}	9.8×10^{5}	3×10^{-1}	9.5×10^{-6}
	3.2×10^{4}	1.2×10^{4}	3.5×10^{-1}	1.2×10^{-5}
Vibrio sp.	2.1×10^{9}	2.4×10^{8}	1.1×10^{-1}	5.4×10^{-11}
	2.1×10^{8}	9.3×10^{6}	4.6×10^{-2}	2.3×10^{-10}
	2.1×10^{7}	6.4×10^{5}	3.3×10^{-2}	1.45×10^{-9}
	2.1×10^{6}	1.8×10^{4}	9.0×10^{-3}	4.1×10^{-9}

Table 32

Influence of initial cell population on the survival of bacterial strains in fish muscle media during freezing and frozen storage at -39±2°C for one month

Bacteril strain	Concentration of cells/ml			
	Initial cell number	Cell number ratio after one month		$K = \frac{(C_2)}{(C_1)^2}$
	C_1	C_2	$\frac{C_2}{C_1}$	
Escherichia coli	11×10^{8}	27×10^{7}	2.4×10^{-1}	2.2×10^{-10}
	11×10^{7}	42×10^{6}	3.8×10^{-1}	3.4×10^{-9}
	11×10^{6}	28×10^{5}	2.5×10^{-1}	2.3×10^{-8}
	11×10^{5}	27×10^{4}	2.4×10^{-1}	2.2×10^{-7}
Pseudomonas sp.	5.6×10^{9}	2.5×10^{9}	0.4×10^{-1}	7×10^{-11}
	5.6×10^{8}	1.07×10^{7}	0.1×10^{-1}	3×10^{-11}
	5.6×10^{7}	9.8×10^{5}	1.75×10^{-2}	3×10^{-10}
	5.6×10^{6}	5.2×10^{4}	1×10^{-2}	1.6×10^{-9}
Micrococcus sp.	1.6×10^{9}	1.4×10^{9}	3.8×10^{-1}	1×10^{-10}
	3.6×10^{8}	9.8×10^{7}	2.72×10^{-1}	7×10^{-10}
	3.6×10^{7}	8.1×10^{6}	2.25×10^{-1}	6×10^{-9}
	3.6×10^{6}	4.2×10^{5}	1.16×10^{-1}	3×10^{-8}
Vibrio sp.	7.2×10^{9}	2.3×10^{9}	2×10^{-1}	4.4×10^{-11}
	7.2×10^{8}	2.2×10^{7}	3×10^{-2}	4.2×10^{-11}
	7.2×10^{7}	6.4×10^{5}	8×10^{-3}	1.2×10^{-11}
	7.2×10^{6}	5.8×10^{4}	8×10^{-3}	1.1×10^{-9}

For E. coli, having an initial cell concentration (C_1) of 10^8 to 10^5/ml, on freezing at -39±2°C in fish muscle, the fraction of the cells surviving namely $\frac{C_2}{C_1}$ was a constant for various initial cell population. But K was not a constant for E. coli. For the other strains namely Pseudomonas, Micrococcus and Vibrio, k was found to be a constant for initial population above 10^8 cells/ml.

At -20±2°C, a constant value for k could be obtained for E. coli when initial cell number was above 10^8 ml. For Pseudomonas, Vibrio and Micrococcus species, even for initial cell population ranging from 10^8 and above, k was not at all a constant.

This study shows that there was no initial population effect of the type described by Major et al. (1954). Eventhough constant 'k' values could be obtained for some strains at higher cell densities, at low cell concentrations the effect vanished.

Major et al. (1955) observed the existence of population effect on the survival during freezing of E. coli, Salmonella gallinarum and Pseudomonas aeruginosa while for Lactobacillus acidophilus, Bacillus coagulans Microbacterium flavum there was no such effect. Postgate, and Hunter (1961) also could not find any such effect with a suspension of Aerobacter aerogenes in 10% glycerol. Record and Taylor (1953) noted that, for 2 strains of E. coli,

survival was proportional to initial cell concentration during freeze-drying and freezing. The results presented here are similar to the observation of Postgate and Hunter (1961) in that the survival was independent of initial cell number at lower dilutions. At higher dilutions, ranging from 10^7 to 10^9 cells/ml, $\frac{C_2}{C_1}$ i.e. fraction of surviving population was related to the initial cell number. As Postgate and Hunter (1961) have suggested, the difference in behaviour could be attributed to the difference in species of test cultures or difference in suspending fluid. According to them, population effect is a phenomenon which manifest on storage in the absence of protective environments. Fish muscle media employed in this study may be the feason for the absence of this effect. The species effect was also noted by Major et al. (1955).

Major et al. (1955) also noted that eventhough the value 'k' was not a constant for some species, there was a relationship between the initial cell number(C_1) and the cells surviving freezing or frozen storage after one month(C_2). They found that the ratio $\frac{C_1}{C_2}$ was found to decrease with decrease in cell densities. In other words, the fraction of cell that survives during freezing or storage improved with increased cell numbers. This type

of behaviour was noted in the present study for cultures E. coli, Pseudomonas and Vibrio at -20±2°C and at -39±2°C and for Pseudomonas and Vibrio at -39±2°C. This shows that survival of these bacteria were better the greater the cell densities. This effect is thought to be due to the protection afforded by the leakage products from injured cells (Ingram and Mackey, 1976). But for Micrococcus sp. at -20±2°C and -39±2°C and for E. coli at -39±2°C, the factor $\frac{C_2}{C_1}$ was almost a constant, showing that survival was independent of initial cell number.

4.3.3.3 Effect of hydrogen ion concentration (pH)

The effect of hydrogen ion concentration on the survival of selected strains of bacteria during freezing and frozen storage are presented in figures 17.1 and 17.2. The results represent the studies conducted at the temperatures viz. -20±2°C and 39±2°C.

On freezing and storage at -20±2°C, E. coli cells with an initial cell concentration of 8×10^8 cells/ml in beef extract adjusted to pH ranging from 3 to 10, showed the highest bacterial count in the pH range of 6-7. At pH 8, there was very slight decrease eventhough it was negligible. In the case of Pseudomonas strain, the peak count was noted at pH 7 - 7.5, whereas for

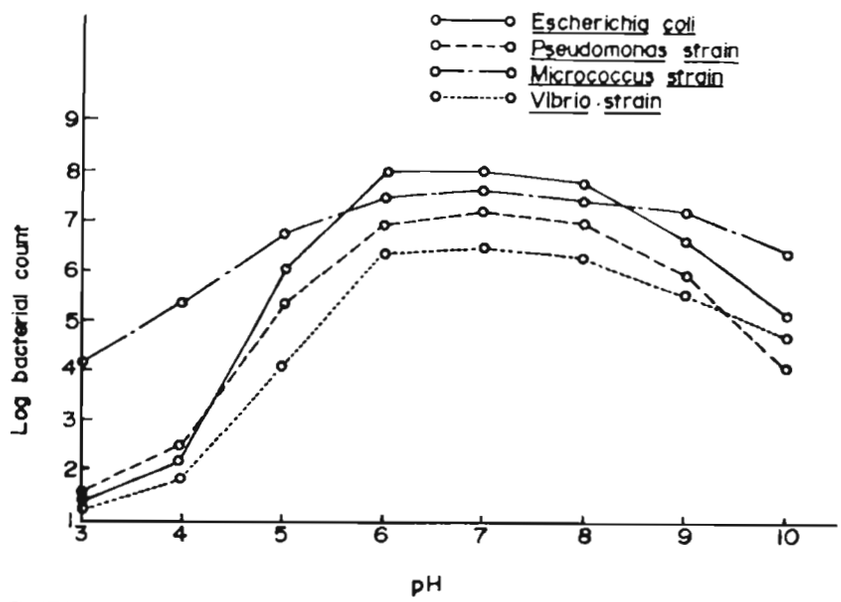

Fig.17.1.
EFFECT OF HYDROGEN ION CONCENTRATION (pH) ON THE SURVIVAL OF SELECTED BACTERIAL SPECIES DURING FREEZING AND STORAGE AT. $-20 \pm 2°C$ FOR ONE MONTH

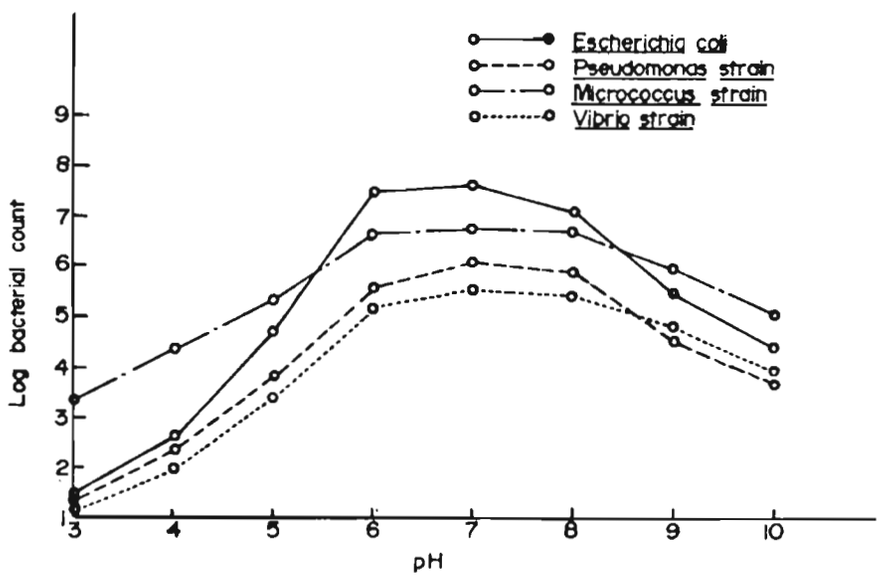

Fig.17.2.
EFFECT OF HYDROGEN ION CONCENTRATION (pH) ON THE SURVIVAL OF SELECTED BACTERIAL SPECIES DURING FREEZING AND STORAGE AT $-39 \pm 2°C$ FOR ONE MONTH

the Micrococcus strain more or less similar values were noted between pH 6 and 7.5, eventhough the highest count was noted at pH 6.5. Vibrio sp., on the otherhand, showed a tendency to survive better at pH range from 7 to 8, But the survivals at pH 6.5 and 8.5 were only slightly less.

At -39±2°C the E. coli cells showed the maximum survival at pH 7.7. For Micrococcus the recovery was almost constant over a wide pH range from 6 to 8 and Pseudomonas strain showed highest survival at pH around 7. The Vibrio sp. could survive at highest pH much better than the other 3 strains.

The beef extract was used in the study because it was difficult to maintain the pH in fish muscle substrate.

A close scrutiny of the figures shows that the strains of E. coli, Micrococcus and Vibrio showed a greater capacity to survive at higher pH. For E. coli the optimum pH at -20±2°C was 6-7 and at -39±2°C they survived at pH 7. For Pseudomonas the same pH afforded maximum survival at both temperatures. These results show that the effect of pH is very significant on survival at low temperatures used for freezing and storage. Between the two temperatures -20±2°C and -39±2°C, there was not much difference.

From this data, it is evident that the pH range during

which maximum survival was noted corresponded to the optimum pH of the test strain and maximum death to acidic pH. This is confirmed by the observation of Georgala and Hurst (1963). They noted that, for Salmonella spp., death was greatest at pH 4.5 and least at pH 6.3, in minced meat at -20°C.

Acidic pH was more harmful according to this study than the alkaline pH for all the organisms studied. For Vibrio sp. a greater capacity to survive in alkaline pH was noted. This is confirmed by the work of Vanderzant and Nickelson (1972) on Vibrio parahaemolyticus. It was found that for Vibrio parahaemolyticus, at low cell concentrations ranging from 10^2 x 10^3 cells/ml, no survivors were noted at pH 1-4. At pH 6-10, eventhough a sharp drop was noticed in early period, the survivors remained constant afterwards.

Among the 3 strains of bacteria, the Gram-positive type Micrococcus spp. showed least susceptibility to changes in pH than Pseudomonas and E. coli, at both -39±2°C and -20±2°C. Demchick et al. (1984) observed that, in meat kept at -20°C, Staphylococcus aureus was not killed at pH values from 4.3 to 6.3.

4.3.3.4 Effect of age of the cells

The data presented in figures 18.1 and 18.2 represents

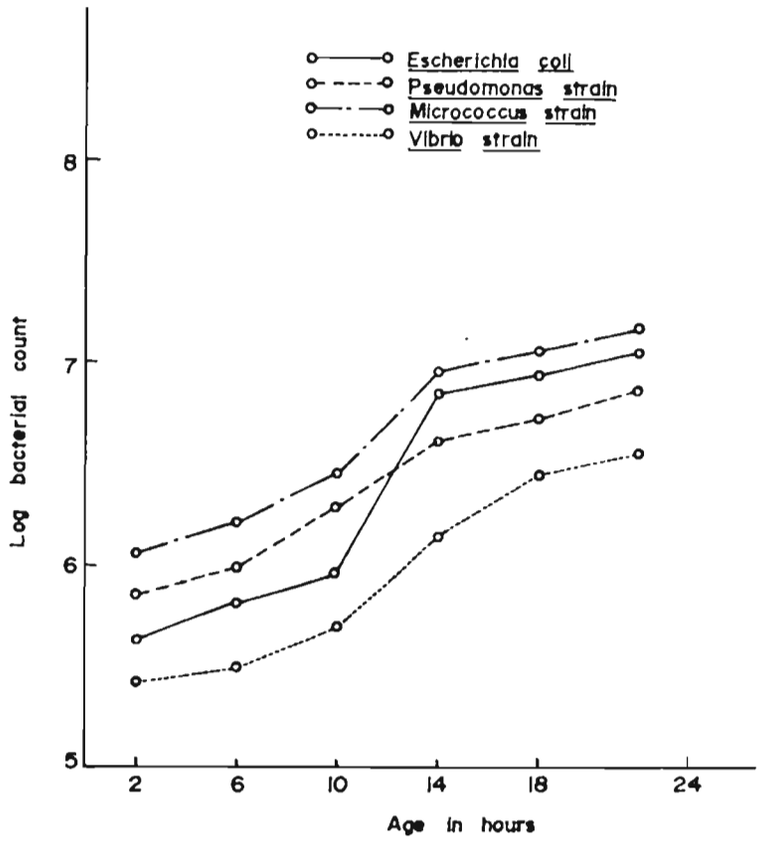

Fig.18.1.
EFFECT OF AGE OF CELLS ON THE SURVIVAL OF SELECTED BACTERIAL SPECIES AFTER STORAGE AT $-20 \pm 2°C$ FOR ONE MONTH

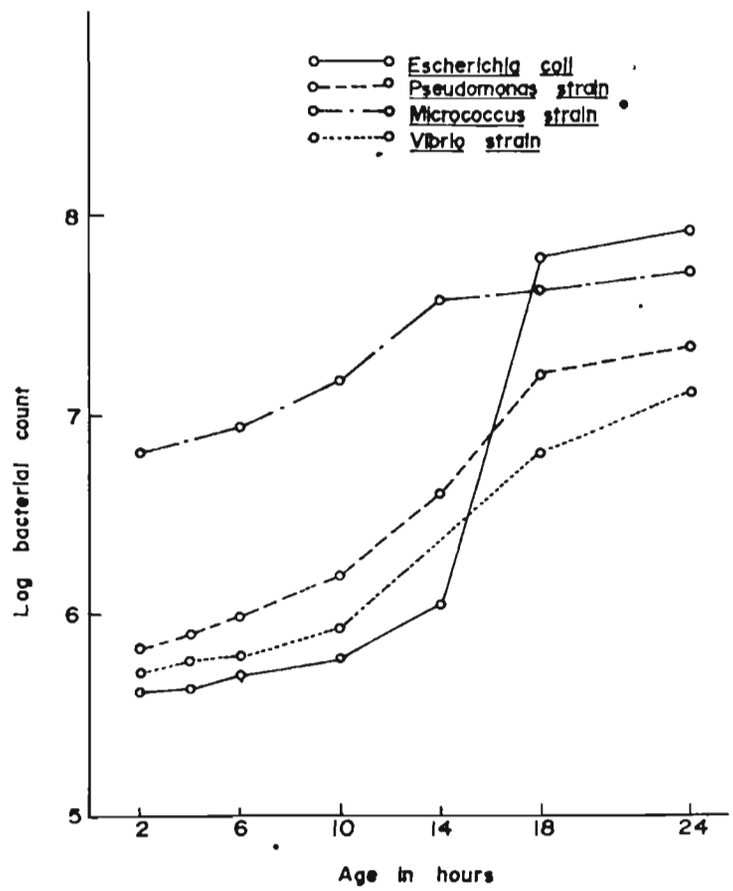

Fig.18.2.
EFFECT OF AGE OF THE CELLS ON THE SURVIVAL OF SELECTED BACTERIAL SPECIES AFTER STORAGE AT $-39 \pm 2°C$ FOR ONE MONTH

the effect of age of cells (phase of growth) on their susceptibility to freezing and frozen storage at temperatures -20±2°C and -39±2°C.

On storage at -20±2°C, the survival rate of all the strains improved with age. For E. coli, there was a sharp increase in survival when the age of the culture was 14 hours and above. The survival of E. coli, with age of 2, 6, 14, 18 and 24 hours, were respectively 26.5%, 28%, 29.8%, 30.5%, 42% and 43% of the initial population. This figure shows that critical change occurred during the period between 10 and 14 hours i.e. corresponding to the middle logarithmic phase.

For Micrococcus also the notable change in survival took place between cells of age 10 hours and 14 hours, but the effect was less significant in this case. The Pseudomonas and Vibrio strains, on the otherhand, showed a gradual improvement in survival with age of cells, especially up to 18 hours i.e. end of logarithmic phase.

On freezing and storage at -39±2°C, some difference was noted in survival rate. While the survival of E. coli cells of 14 hour of age was 30%, for cells of 18 hours age, survival was 47.5%. But this sharp rise occurred only in the case of E. coli. For other cultures the trend was more or less same at both temperatures.

The survival rates as percentage of the initial population of 24 hour old Pseudomonas, E. coli, Micrococcus and Vibrio strains at -20±2°C were respectively 38.5, 40.5, 42 and 35.5. The survival of the same strains with identical initial cell concentration showed survival rates as 43%, 48%, 47% and 41% when stored at -39±2°C. This showed that resistance of the stationary phase cells were lower at -20±2°C than at -39±2°C. (Fig.18.1 and 18.2)

These results thus indicate that cells in the logarithmic phase are more sensitive than stationary phase cells to the lethal action of freezing and storage at -39±2°C and -20±2°C in fish muscle. Similar behaviour for bacteria exposed to freezing (Toyokama and Hollander, 1956) and chilling temperatures (Strange et al., 1961) are documented.

4.3.3.5 Effect of the presence of some chemicals on survival of bacteria during freezing and frozen storage

4.3.3.5.1 Effect of sucrose

The figures 19.1 and 19.2 represent the effect of added sucrose on the viability and survival of selected strains of bacteria. The effect of freezing and storage at two temperatures viz. -20±2°C and -39±2°C were studied.

Over a sucrose concentration ranging from 0.5% to

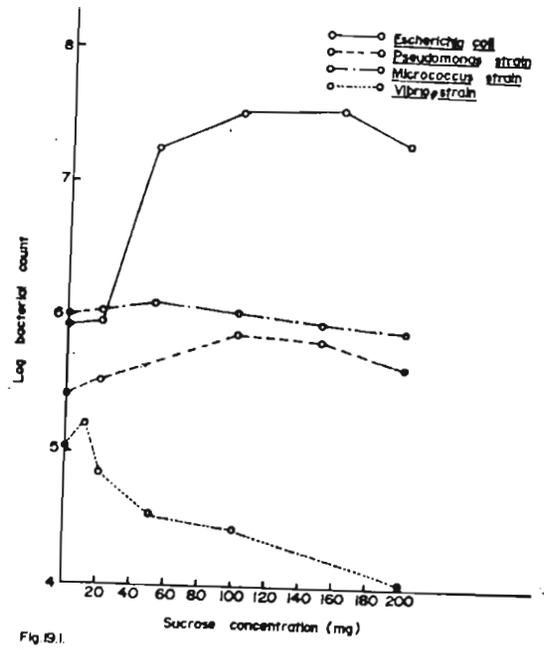

Fig 19.1.
EFFECT OF SUCROSE ON THE SURVIVAL OF SELECTED BACTERIA AFTER STORAGE AT -20 ± 2°C FOR ONE MONTH

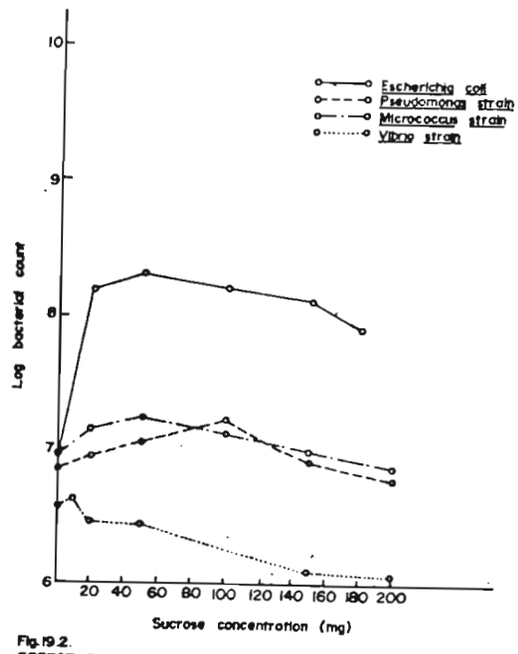

Fig. 19.2.
EFFECT OF SUCROSE ON THE SURVIVAL OF BACTERIA AFTER STORAGE AT -39 ± 2°C FOR ONE MONTH

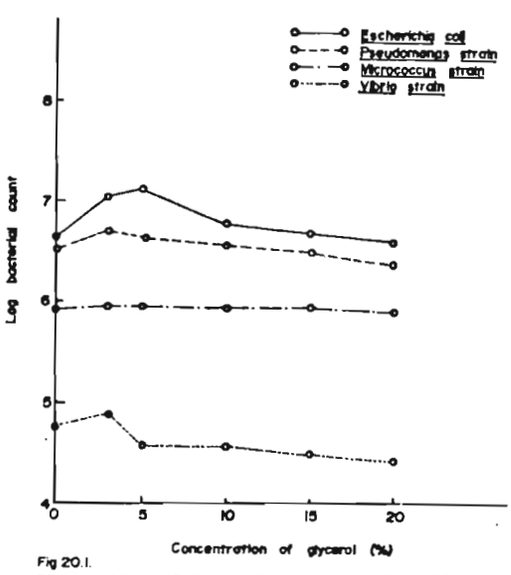

Fig 20.1.
EFFECT OF GLYCEROL ON THE SURVIVAL OF SELECTED BACTERIA AFTER STORAGE AT -20 ± 2°C FOR ONE MONTH

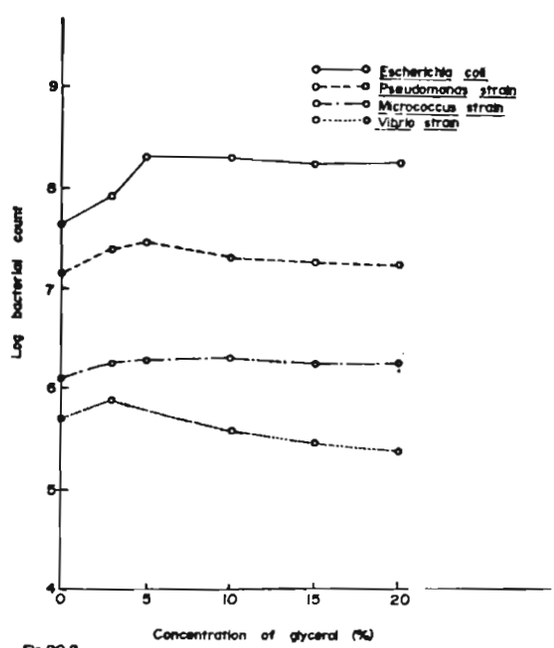

Fig. 20.2.
EFFECT OF GLYCEROL ON THE SURVIVAL OF SELECTED BACTERIA AFTER STORAGE AT -39 ± 2°C FOR ONE MONTH

20% i.e. 5 mg/ml to 200 mg/ml in distilled water, very significant results were noted. E. coli cells, with an initial cell density of 2.9×10^9 cells/ml (count at room temperature $29 \pm 2°C$), when suspended in the sucrose solution and frozen stored at $-20 \pm 2°C$ showed an improvement in survival up to 2% i.e. 20 mg/ml. There was very little effect thereafter on the rate of survival and a maximum survival was noted for a sucrose concentration between 100-160 mg/ml. The minimum concentration of sucrose to effect protection was found to be 20 mg/ml. Increase in sugar concentration over 16% i.e. 160 mg/ml was found to affect the survival adversely.

The initial population of Pseudomonas sp. used in this study was 7.34×10^6 cells/ml. In this case the effect was not so pronounced as that of E. coli. Still, an improvement in count was observed by suspending the cells in sucrose solution. Maximum survival corresponded to a sucrose concentration of 100 mg/ml i.e. 10%.

The initial cell count used in the case of Vibrio sp. was 1.94×10^7 cells/ml. There was only a transient improvement in survival in this case and the sucrose concentration at which maximum survival was noted corresponded to 10 mg/ml. Increasing the concentration of sucrose above 10 mg/ml had a deleterious effect on survival.

The Micrococcus sp., having an initial count of 3.2×10^6 cells/ml, when suspended in the sucrose solution and frozen, showed very little effect. Still, a peak value in survival was noted corresponding to a sucrose concentration of 50 mg/ml.

During storage of these cultures in sucrose solution at $-39 \pm 2^{\circ}C$ for one month (Fig.19.2), the trend was more or less same for the strains. Exception was noted in the case of E. coli, where the concentration of sucrose corresponding to maximum survival was 5% (50 mg/ml). For Pseudomonas sp., the maximum survival at both temperatures corresponded to 100 mg/ml. The initial cell count of E. coli, Pseudomonas sp., Micrococcus sp. and Vibrio sp. in this case were 2.9×10^8 cells/ml, 7.34×10^8 cells/ml, 1.33×10^8 cells/ml and 4.58×10^7 cells/ml respectively.

This study showed that sucrose had protective effect on bacterial cells during freezing and frozen storage, which depended on the concentration of sugar also. While E. coli exhibited maximum protection in sucrose, the effect was less significant for Micrococcus sp. and for Vibrio sp. there was least protection.

4.3.3.5.2 Effect of glycerol

The initial cell count of E. coli, Pseudomonas sp.

Micrococcus sp. and Vibrio sp. used for freezing and frozen storage at -39±2°C were respectively 2.9×10^8 cells/ml, 3.22×10^8 cells/ml, 4.81×10^7 cells/ml and 9.24×10^7 cells/ml. At -20±2°C, the densities of the initial inoculum of these cultures were 2.9×10^8 cells/ml 4.89×10^7 cells/ml 1.56×10^6 cells/ml and 1.08×10^7 cells/ml. Survival of selected microorganisms during freezing and storage at the temperatures -20±2°C and -39±2°C are represented by figures 20.1 and 20.2.

It is seen from the figures that glycerol also had a similar effect as that of sucrose in protecting the cells during freezing and frozen storage. At -39±2°C, the concentration of glycerol affording maximum protection for E. coli was found to be 5%. The same level of glycerol afforded protection for the Pseudomonas sp. also, while for Vibrio sp., 3% glycerol was found to effect maximum protection. For Micrococcus sp., a concentration of glycerol exceeding 3% was found to have little effect.

On storage of these bacterial cultures at -20±2°C, more or less similar behaviour was noted for all cultures. Exception was only in the case of Pseudomonas sp. for which the concentration of glycerol affording maximum protection was found to be 3%.

It is also evident from the figures that eventhough both glycerol and sucrose had a protective effect on

bacterial cells, the pattern of survival on increasing the levels of the two were entirely different. After reaching the limit, increasing concentration of sucrose resulted in fall in bacterial count and decrease in the survival. But in the case of glycerol, the higher concentrations did not improve the survival but just maintained it. In other words, increasing concentration of glycerol beyond certain level was ineffective, but not disastrous to cells.

4.3.3.5.3 Effect of salts

The effect of presence of sodium chloride and potassium chloride on the survival of selected species of bacteria during freezing and frozen storage at temperatures -20±2°C and -39±2°C are illustrated in Figures 21.1 and 21.3. Initial count of E. coli, Pseudomonas sp. Vibrio sp. and Micrococcus sp. were 1.31×10^9, 9.3×10^7, 1.35×10^8 and 2.56×10^8 cells/ml respectively.

When E. coli cells were placed in varying concentration of sodium chloride in distilled water, frozen and stored at -39±2°C, the bacterial count remained almost stationary up to 20 mg/ml i.e. 2% after which there was a sudden drop. For Pseudomonas sp., the survival remained almost constant up to 5% Nacl, after which there was a progressive fall in survival rate. The Vibrio sp. showed

a gradual decrease in the survival even from the beginning. On the otherhand, Micrococcus sp., after reaching peak survival corresponding to sodium chloride concentration of 2%, showed only very slight decrease in survival with increasing salt concentration.

At -20±2°C, all the cultures showed similar trend as at -39±2°C. The cell densities of these bacteria on initial sampling were found to be 2.3×10^8 cells/ml for E. coli, 5.85×10^7 cells/ml for Micrococcus sp., 1.17×10^7 cells/ml for Vibrio sp. and 6.46×10^7 cells/ml for Pseudomonas sp.

Potassium chloride exhibited an effect similar to that of sodium chloride (Fig. 22.1 and 22.2). Frozen storage of E. coli cells having an initial count of 1.21×10^9 cells/ml in varying concentration of potassium chloride in distilled water at -39±2°C for one month (Fig.22.2) resulted in drastic fall in survival rate above 0.5% Kcl. For Pseudomonas (initial count of 6.88×10^7 cells/ml) a similar fall in survival occurred after 1% Kcl. While Micrococcus sp. (initial count of 1.08×10^8 cells/ml) showed no decrease at all, Vibrio sp. (initial count of 4.22×10^8 cells/ml) registered a fall in count from the beginning.

At -20±2°C E. coli, Pseudomonas sp. and Vibrio sp.

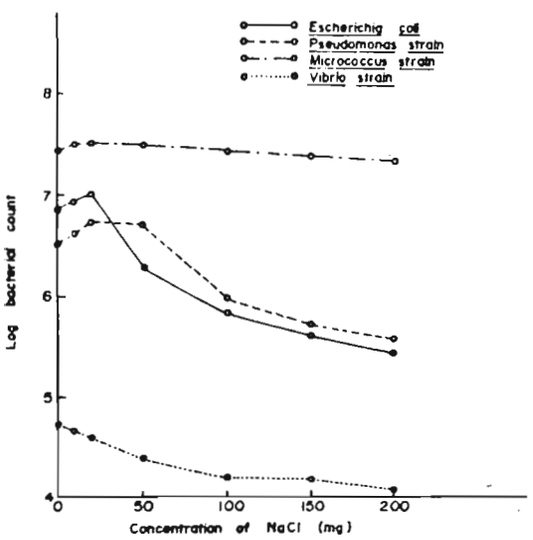

Fig 21.1.
EFFECT OF NaCl ON THE SURVIVAL OF SELECTED BACTERIA AFTER STORAGE AT −20 ± 2°C FOR ONE MONTH

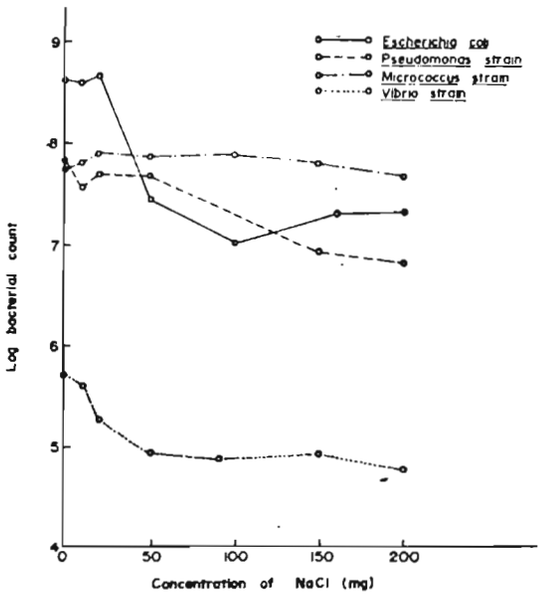

Fig.21.2
EFFECT OF NaCl ON THE SURVIVAL OF SELECTED BACTERIA AFTER STORAGE AT −39 ± 2°C FOR ONE MONTH

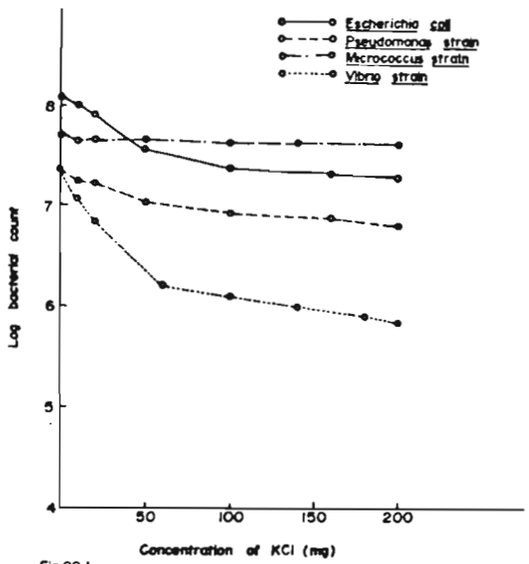

Fig.22.1.
EFFECT OF KCl ON THE SURVIVAL OF SELECTED BACTERIA AFTER STORAGE AT −20 ± 2°C FOR ONE MONTH

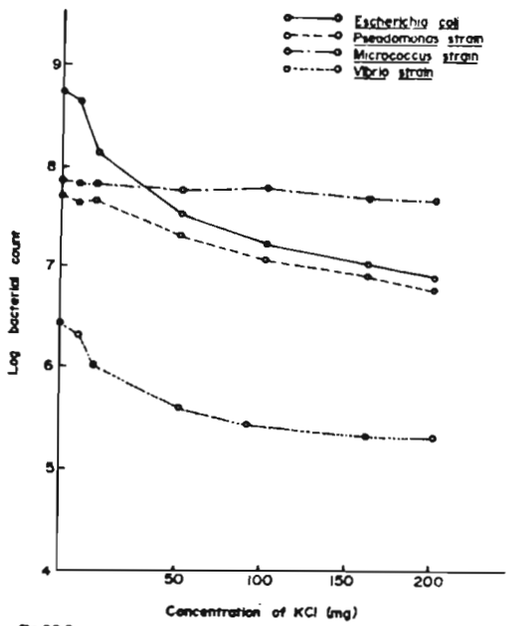

Fig.22.2.
EFFECT OF KCl ON THE SURVIVAL OF SELECTED BACTERIA AFTER STORAGE AT −39 ± 2°C FOR ONE MONTH

showed a gradual decrease in survival rate. For Micrococcus sp. the effect was less significant. The initial cell count used in the case of E. coli, Pseudomonas sp., Micrococcus sp. and Vibrio sp. were respectively 1.31×10^7 cell/ml, 2.34×10^8 cells/ml, 9.86×10^7 cells/ml and 2.3×10^9 cells/ml (Fig.22.1)

These results indicated that the salts, sodium chloride and potassium chloride had a harmful effect on the viability of bacterial cells. Except Vibrio sp. the survival of other strains were slightly benefited by very low levels of sodium chloride. But in the case of Kcl, even low levels were found to be harmful to these bacteria. According to Drapeau and MacLeod (1965), Kcl is less effective than Nacl in preventing the loss of solutes from the cell. The observed difference may be due to this variation.

According to Lovelock (1953), the harmful effect of low temperature is the high salt concentration within the cell cytoplasm due to crystallisation of water. Mazur (1960) stated that sugar and ethylene glycol protected the cell by preventing or reducing the likelihood of intra-cellular freezing by preventing the seeding of the ice crystal through the cell membrane. Intra-cellular freezing is supposed to be pre-requisite for death at subzero temperature Mazur (1960). Further proof of this theory was provided by Lusena and Cook (1953) who found

that ice crystals from distilled water penetrated through 0.05 mm hole in the otherwise impermeable polystyrene sheet whereas ice crystals from 0.1 M sucrose did not penetrate 3.0 mm hole. 0.1 M Kcl also did not penetrate 3 mm opening. The two substances sucrose and glycerol differed in their penetration properties glycerol being more penetrative than sucrose or glucose. The difference in the survival of cells observed here may be attributed to the difference in penetrative properties. According to Postgate and Hunter (1961), penetration is irrelevant to protection against killing by freezing.

The protective action of sucrose and glycerol has been noted by many workers. Calcott and MacLeod (1974) observed that for E. coli the minimum concentration of sucrose that afforded protection at -70°C was 5%. 2.5% gave intermediary protection at cooling rate of 5°C/min. Similarly, glycerol exerted a protective effect on a population of E. coli at a concentration of 3% or more. In sucrose viability decreased with storage period. After storage for 2, 8 and 24 hours at -20°C the survival was 78, 60 and 25% respectively (Postgate and Hunter, 1961). In addition to the above agents, others such as ethylene glycol, glucose, lactose etc. have been reported to afford protection to bacteria during freezing (Mazur, 1960). The present study confirm the above finding, though

slightly higher value was noted for E. coli at -39±2°C.

The increased salt concentration decreases the water activity (a_w) within the cell. Salts caused dehydration and loss of solutes from the cell. The effect of added salt, according to Ingram and Mackey (1976), is to lower the freezing point of the system with the result that cells are exposed to more concentrated solution and greater osmotic stress, for longer period. Temmyo (1966) found that Nacl in concentration ranging from 0.5% to 7% appeared to give some protection to Vibrio parahaemolyticus. However, this effect was not present during long term storage. Sodium chloride was found to have a protective effect (3% level) on Vibrio parahaemolyticus held at -48°C for 60 min or -18°C for 30 days (Covert and Woodburn, 1972). But the Vibrio strain selected in this study behaved in a different way. It experienced no effect from the salt. Probably the difference in the behaviour may be due to the difference in the species or the freezing menstruum. Also, as stated by Temmyo (1966), the storage at low temperatures for one month may be the reason for this behaviour. As seen in previous chapter (3.1.1), more than 99% of the Vibrio species are killed by storage at -39±2°C or -20±2°C for one month.

The Micrococcus group showed particular ability to resist high salt concentration. The water requirement of

this group has been subjected to a thorough study (Scott, 1953; Christian and Waltho, 1961) and it has been clearly established that the group possessed low water requirements. Hence, the greater resistance of the Micrococcus group to high salt concentration could be due to its ability to thrive on comparatively lower a_w.

4.3.4 Characterization of the injury induced by freezing and frozen storage of selected bacterial strains

4.3.4.1 Effect of freezing menstrua on injury

The survival pattern of selected strains of bacteria in different suspending fluids at -20±2°C are presented in Table 33. The strains included in this study were Pseudomonas, E. coli and Micrococcus isolated from fish. Results presents average of three series.

As seen from the data for E. coli, lowest death at -20±2°C was in fish muscle medium being 63% of the initial population. While 15% were injured, 23% of cells proved to be unharmed. Beef extract (1%) proved to be almost equal to fish muscle in protection, the percentage of dead, injured and unharmed cells being 68 , 13 and 19. Greatest death was observed in distilled water (88%) and only 1% of cells came out as unharmed.

Table 33

Effect of suspending fluids on the death and injury on selected bacterial strains during freezing*

Substrate	*Escherechia coli*			*Pseudomonas* sp.			*Micrococcus* sp.		
	Percentage cells of initial population			Percentage cells of initial population			Percentage of cells of initial population		
	Unharmed	Dead	Injured	Unharmed	Dead	Injured	Unharmed	Dead	Injured
Normal saline	4	72	24	15	65	24	25	43	32
Distilled water	1	88	11	3	67	30	30	55	15
Fish muscle medium	23	62	15	24	55	21	40	37	23
Beef extract	19	68	13	24	57	19	38	36	26
Phosphate buffer	17	75	8	17	68	13	33	45	22

*Bacterial cells were frozen and stored at -20±2°C for one month

For Pseudomonas also, lowest death of cells occurred when suspended in fish muscle medium (55%) and highest in phosphate buffer (68%). It should be pointed out that more cells were in the injured state for Pseudomonas than E. coli. Injury was more in the case of distilled water and the percentage of injured E. coli and Pseudomonas cells in distilled water were 11 and 30 while the percentage of unharmed were 1 and 3 respectively.

The Micrococcus sp. showed almost identical death in fish muscle medium and beef extract solution. Highest death of 55% was obtained in distilled water. Death was comparable in normal saline and phosphate buffer, but the injury was less in phosphate buffer compared to normal saline.

This study showed that extent of death and injury depended on the nature of the suspending fluid. Among the suspending fluids studied here, fish muscle media was most protective and distilled water least so as shown by a high percentage of unharmed cells and low percentage of dead ones.

4.3.4.2 Effect of storage temperature

The effect of storage temperature and the storage period on the survival pattern of these bacteria are illustrated by Tables 34 and 35.

Table 34

Effect of freezing and storage at -20±2°C on the death and injury of selected bacterial strains

Storage time	*Escherechia coli*			*Pseudomonas* sp.			*Micrococcus* sp.		
	Percentage of cells as			Percentage of cells as			Percentage of cells as		
	Unharmed	Dead	Injured	Unharmed	Dead	Injured	Unharmed	Dead	Injured
6 h	38	24	38	41	20	39	44	11	45
1 day	24	39	31	33	31	36	38	20	42
7 days	21	51	28	29	42	29	29	32	39
15 days	14	60	26	22	52	26	33	37	40
1 month	13	65	22	20	57	23	26	40	34

Table 35

Effect of freezing and storage at -39±2°C on death and injury of selected bacterial strains

Storage period	*Escherechia coli*			*Pseudomonas* sp.			*Micrococcus* sp.		
	Percentage of cells as			Percentage of cells as			Percentage of cells as		
	Unharmed	Dead	Injured	Unharmed	Dead	Injured	Unhared	Dead	Injured
6 h	35	28	37	43	21	37	41	9	50
1 day	30	44	26	33	39	28	36	18	46
7 days	29	50	21	28	49	23	24	33	43
15 days	15	60	25	26	51	23	25	34	41
30 days	13	63	24	24	55	21	23	37	40

At -39±2°C, for E. coli the percentage of dead cells after 6 hours were 21, in fish muscle medium which increased to 39 after 1 day, 49 after 7 days and 59 after 1 month. There was a progressive increase in dead cells. Corresponding decrease in the percentage of unharmed and injured cells occurred simultaneously. It could also be seen that greater death occurred in the early period of storage. This trend was observed for all the three strains under study.

The data brought out some salient points regarding the storage temperature on survival pattern. The percentage of dead cells at -39±2°C and -20±2°C after storage for 1 month were respectively 55 and 57 for Pseudomonas sp., 63 and 65 for E. coli and 37 and 40 for Micrococcus. But the percentage of injured cells at -39±2°C and -20±2°C for Pseudomonas sp. were 21 and 23, for E. coli 24 and 22 and for Micrococcus sp. 23 and 26 respectively. Corresponding values of unharmed cells for Pseudomonas spp. were 24 and 20, E. coli 24 and 13 and Micrococcus sp. 40 and 34 respectively. This shows that the percentage of dead and injured cells remained almost same at the two temperatures.

Within a specified temperature the loss of viability or death was greatest for E. coli, being 63% at -39±2°C. For Pseudomonas sp., it was 55% and for Micrococcus sp., 37%.

This showed that E. coli was more sensitive than Pseudomonas sp. which in turn was more sensitive than Micrococcus sp. A Pseudomonas species was reported to be more resistant than E. coli (Arpai, 1962). According to Straka and Stokes (1959), the percentage of dead cells of E. coli were not much different from the Pseudomonas. The percentage of injured cells in Pseudomonas fluorescens, Pseudomonas ovalis and Pseudomonas geniculata were respectively 25, 27 and 17, whereas percentage of dead were 56, 38 and 61 respectively. According to the work of Jackson (1974), Staphylococcus aureus and E. coli were injured by cold, but Streptococcus fecalis was not affected. But the data of Moss and Speck (1963) on Streptococcus lactis showed that the cells were metabolically injured. 50% of the initial population were killed by fast freezing in distilled water and 82% by slow freezing at temperature from +20°C to -35°C.

Death and injury was more significant in the early period. For Pseudomonas sp. stored at -39±2°C, an increase was noted in the percentage of dead cells. The percentage of dead cells increased on storage. Similar observation was reported by Haines (1938), Gunderson and Rose (1948), Straka and Stokes (1959), Arpai (1962), Moss and Speck (1963) and Jackson (1974). However, injury decreased continously as was observed here. Since injury leads

to death, fewer cells are left back to be injured so that the percentage of injured decreases (Straka and Stokes, 1959).

Arpai (1962) noted a decrease in the injured cells of Pseudomonas fluorescens during 1 to 7 days and an increase from 7 to 15 days in most cases. However, the percentage of death increased.

But Moss and Speck (1963) noted a continuous decrease in the injured cells and an increase in the dead cells with increase in storage period.

Regarding the temperature of freezing and storage, Straka and Stokes (1959) reported that lowering of temperature from -7 to -29°C caused an increase in the percentage of injured cells and a decrease in dead cells. They attributed this inverse relationship to the fact that extensive death left fewer cells in the intermediary stage of injury. The data, reported by Arpai (1962), showed that freezing and storage of Pseudomonas fluorescens in meat extract at pH 7 and temperatures -7°C, -28°C and -30°C for 15 days resulted in the injury of 40, 40 and 31%. Similar values for E. coli were 35, 28 and 20%. The percentage of dead cells at these temperatures for Pseudomonas fluorescens were 25, 41 and 51 and that of E. coli 43, 64 and 74. This showed an increase in the percentage of dead cells

and decrease in injured cells. The present data corroborates more with that of Arpai (1962).

According to Straka and Stokes (1959), the injury is inversely proportional to the death. Since in the present study death was more or less equal at two temperatures, injury should be same. This was found to be in agreement to observation of Straka and Stokes (1959).

The present work showed that the suspending fluid had a marked effect on death and injury of the suspended cells during freezing and storage. Similar observation was recorded by other workers also, Straka and Stokes (1959) noted extensive death of E. coli in 1% peptone water and full protection against injury in 10% skim milk. The other fluids tested by them were yeast extract, beef extract and distilled water. A comparison of different freezing menstruam on the survival of Pseudomonas and E. coli showed Skim milk to be most protective and distilled water least so (Arpai, 1962). The study of Moss and Speck (1963), on Streptococcus lactis, showed that injury and death was more in distilled water than 10% non fat skim milk. The present study shows that fish muscle compares very well with skim milk with regard to protective action.

4.3.5 Growth of bacteria during thawing

Figures 23 to 31 show the effect of thawing temperatures on the growth and multiplication of selected strains of bacteria after freezing and storage in fish muscle media (FMM) and nutrient/sea water peptone broth at -39±2°C for 1 month. The control consisted of unfrozen cells of the same strains in approximately equal concentration prepared as outlined in 3.2.6.1. These were suspended in the same media or substrate and kept at temperatures +2°C, +15°C and RT (29±2°C).

The frozen cells of Escherichia coli, when allowed to thaw at +2°C, +15°C and RT (29±2°C) showed maximum growth at RT. At +15°C, growth rate was slower than that of RT. At +2°C, practically little growth was noticed. The growth curves obtained at RT for frozen cells were almost identical to the above growth curve obtained for unfrozen cells at RT. But a slight lag in growth phase in the early period up to 6 hours observed in the case of frozen cells was absent in the case of control.

On thawing at +15°C, frozen cells of Pseudomonas exhibitedalag as observed in the figure 24. Though a slight lag in growth was observed in the case of unfrozen cells also, the lag of control cells were much less than that of frozen cells. The total count of control strain

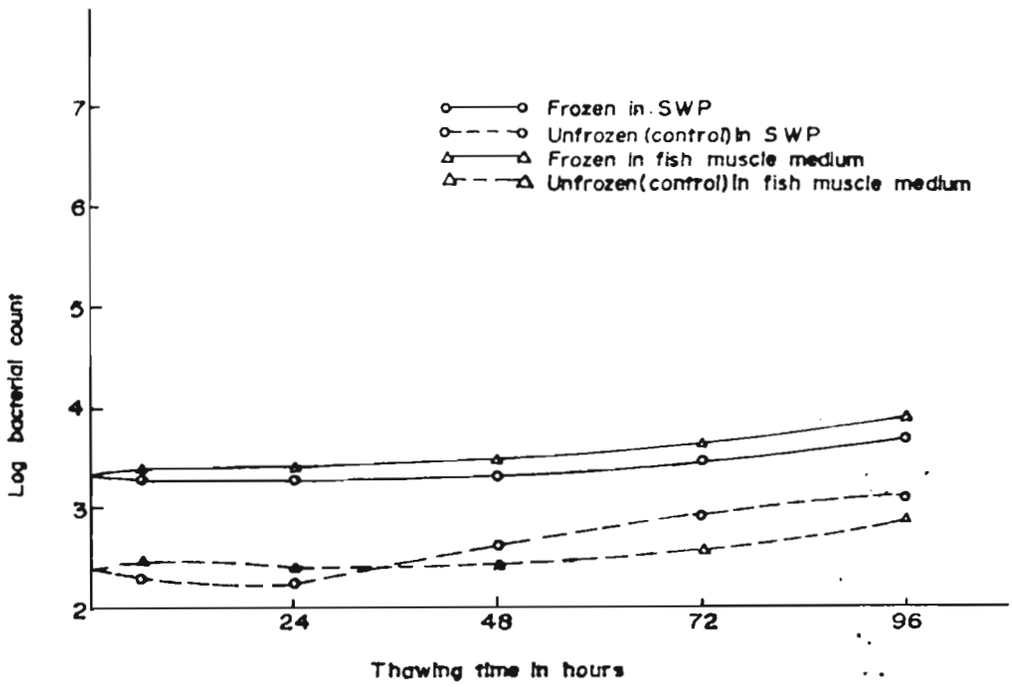

Fig.23.
INCREASE IN THE POPULATION OF A PSEUDOMONAS STRAIN DURING THAWING A+2°C AFTER FREEZING AT-39±2°C FOR 6 HRS. AND FROZEN STORAGE AT-20±2°C FOR ONE MONTH

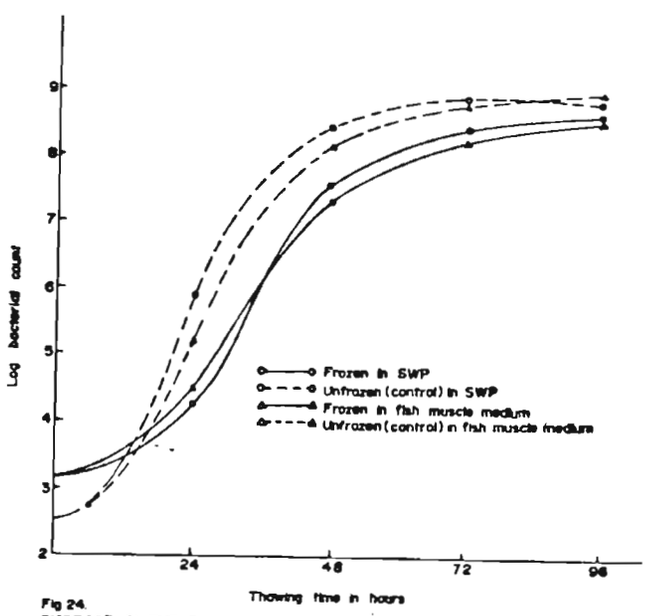

Fig 24.
INCREASE IN THE POPULATION OF A PSEUDOMONAS STRAIN DURING THAWING AT +15°C AFTER FREEZING AT-39± 2°C FOR 6HRS. AND FROZEN STORAGE AT-20±2°C FOR ONE MONTH

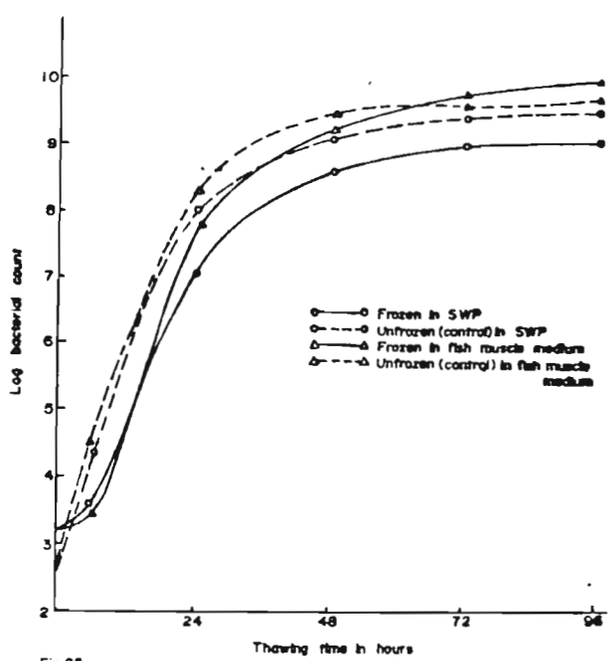

Fig 25.
INCREASE IN THE POPULATION OF A PSEUDOMONAS STRAIN DURING THAWING AT 29±2°C AFTER FREEZING AT-39±2°C FOR 6HRS. AND FROZEN STORAGE AT-20±2°C FOR ONE MONTH

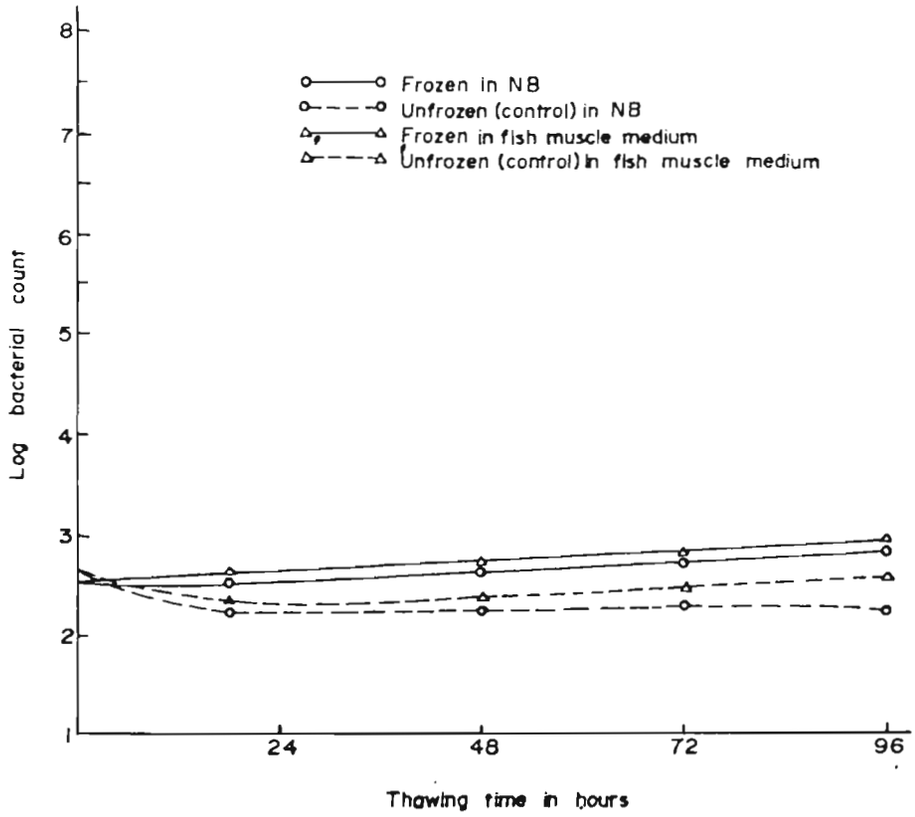

Fig.26.
INCREASE IN THE POPULATION OF AN ESCHERICHIA COLI STRAIN DURING THAWING AT +2°C AFTER FREEZING AT -39±2°C FOR 6HRS. AND FROZEN STORAGE AT -20±2°C FOR ONE MONTH

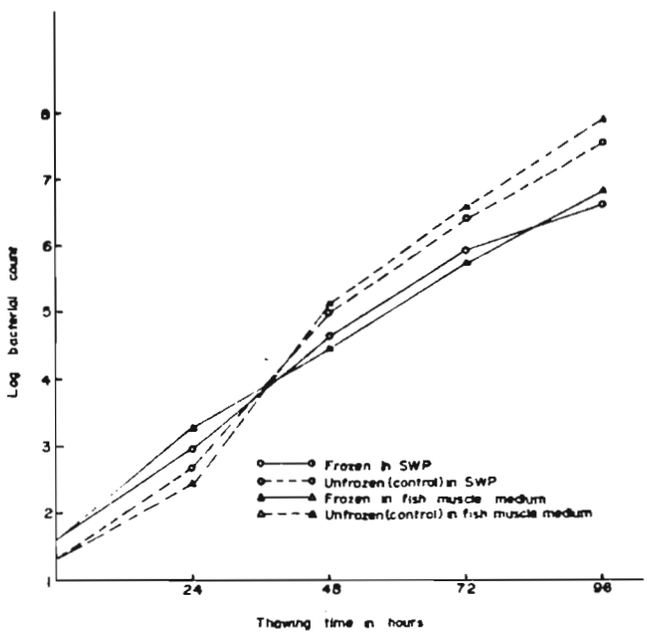

Fig.27
INCREASE IN THE POPULATION OF AN ESCHERICHIA COLI STRAIN DURING THAWING AT +15°C AFTER FREEZING AT -39±2°C FOR 6HRS AND FROZEN STORAGE AT -20±2°C FOR ONE MONTH

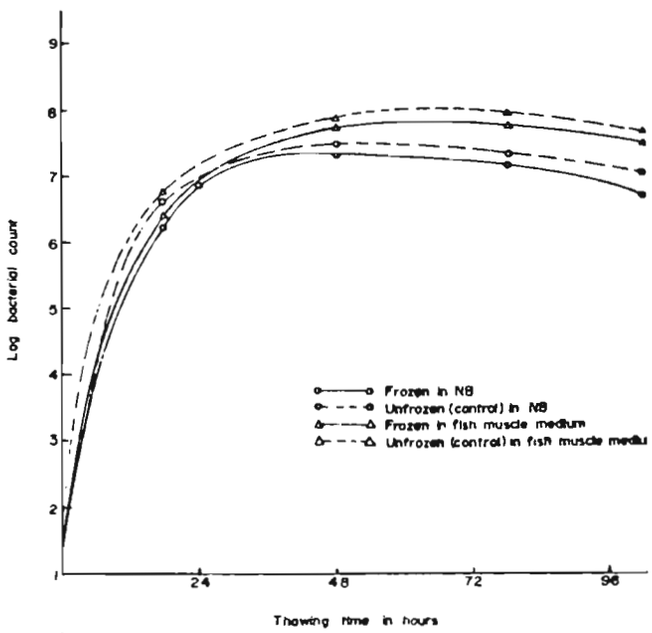

Fig 28
INCREASE IN THE POPULATION OF AN ESCHERICHIA COLI STRAIN DURING THAWING AT 29±2°C AFTER FREEZING AT -39±2°C FOR 6HRS. AND FROZEN STORAGE AT -20±2°C FOR ONE MONTH

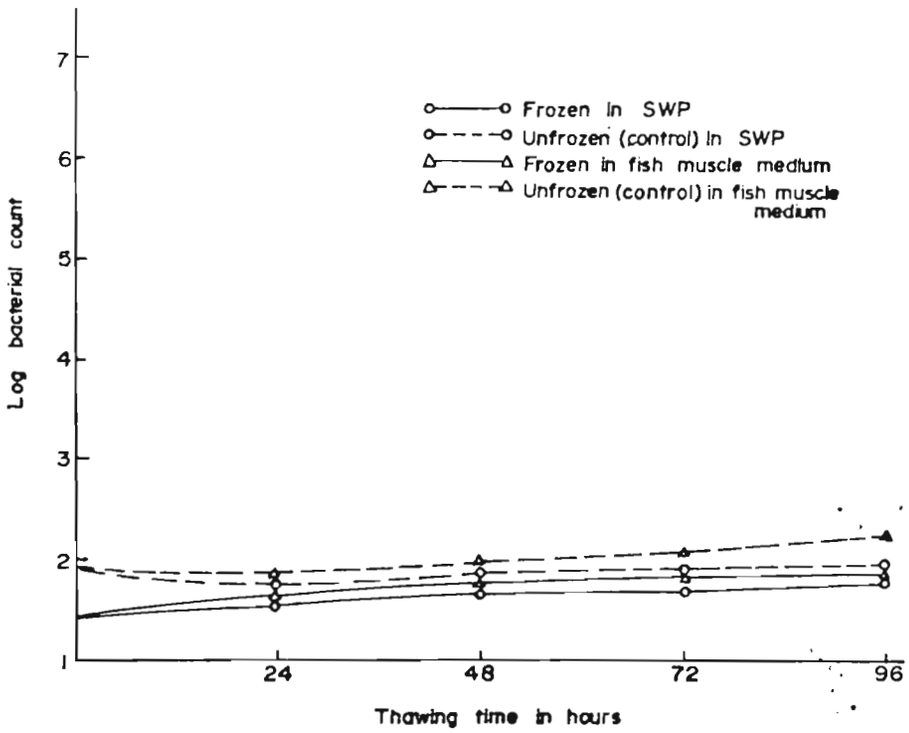

Fig.29.
INCREASE IN THE POPULATION OF A MICROCOCCUS STRAIN DURING THAWING AT +2°C AFTER FREEZING AT −39±2°C FOR 6HRS AND FROZEN STORAGE AT −20±2°C FOR ONE MONTH

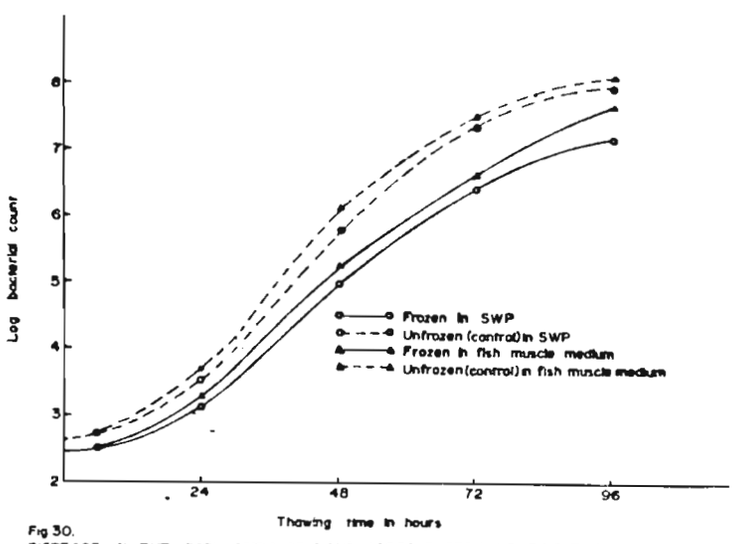

Fig 30.
INCREASE IN THE POPULATION OF A MICROCOCCUS STRAIN DURING THAWING AT +15°C AFTER FREEZING AT −39±2°C FOR 6HRS AND FROZEN STORAGE AT −20±2°C FOR ONE MONTH

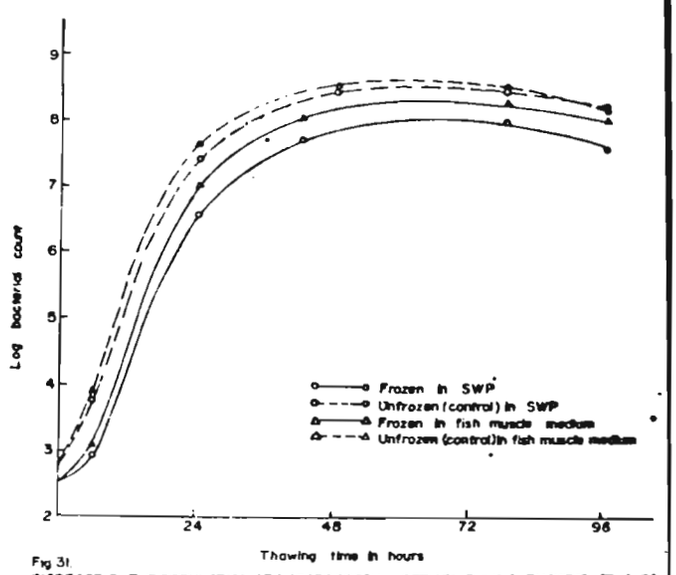

Fig 31.
INCREASE IN THE POPULATION OF A MICROCOCCUS STRAIN DURING THAWING AT +15°C AFTER FREEZING AT −39±2°C FOR 6HRS. AND FROZEN STORAGE AT −20±2°C FOR ONE MONTH

exceeded that of frozen culture after 24, 48, 72 and 96 hours of thawing. However, the difference between the two counts diminished with progress of thawing.

The cell numbers of the control and frozen Pseudomonas continued to increase up to 96 hours at +15°C. E. coli also behaved similarly. But in the case of Micrococcus strain, the control showed lowered multiplication rate than the frozen cells in the early period up to 42 hours. But after that the unfrozen cells showed greater growth rate than frozen.

On thawing at +2°C, the Pseudomonas strain almost maintained their cell numbers both in relation to frozen cells and control cells. Eventhough the initial inoculum of unfrozen cells slightly exceeded the frozen cells, the difference was maintained throughout the thawing period indicating identical growth for control and frozen cells at +2°C. In the case of E. coli, the growth curve of frozen cells remained almost stationary, while that of control showed slight decrease. In the case of Micrococcus strain, the frozen cells maintained their numbers, while the control showed decrease in the early period (up to 96 hours).

The generation times calculated for these strains at different temperatures of thawing are given (Tables 36.1 to 36.3)

Table 36.1

Generation time of Escherichia coli strain during intervals of thawing at different temperatures in fish muscle

Intervals of thawing/hours	Frozen cells on thawing at			Unfrozen cells (control) kept at		
	+2°C	+15°C	RT (29±2°C)	+2°C	+15°C	RT (29±2°C)
0 - 24	73.0	4.38	1.48	-	6.29	1.34
24 - 48	72.2	6.28	5.00	-	2.72	9.20
48 - 72	76.0	5.16	180.0		5.78	145.00
72 - 96	37.0	7.72	-	72.0	5.35	

Table 36.2

Generation time of Pseudomonas strain during intervals of thawing at different temperatures in fish muscle

Intervals of thawing/hours	Frozen cells on thawing at			Unfrozen cells (control) kept at		
	+2°C	+15°C	RT (29±2°C)	+2°C	+15°C	RT (29±2°C)
0 - 24	36.14	3.35	1.60	72.20	2.78	1.26
24 - 48	48.19	2.58	5.17	72.20	2.40	6.00
48 - 72	48.19	8.03	7.20	72.20	12.04	24.20
72 - 96	30.00	24.09	26.60	26.60	36.14	72.20

Table 36.3

Generation time of Micrococcus during intervals of thawing at different temperatures in fish muscle

Intervals of thawing/hours	Frozen cells on thawing at			Unfrozen cells (control) kept at		
	+2°C	+15°C	RT (29±2°C)	+2°C	+15°C	RT (20±2°C)
0 - 24	500.00	8.50	1.68	-	6.57	1.49
24 - 48	150.00	3.61	7.20	-	2.83	8.00
48 - 72	772.20	5.16	-	93.70	5.78	-
72 - 96	80.53	6.57	-	100.00	12.05	-

For frozen cells of Pseudomonas, E. coli and Micrococcus, the lowest generation times were observed at RT corresponding to the first 24 hours of thawing. At +15°C, the lowest generation time was in 24 - 48 hours for frozen Pseudomonas and Micrococcus strains and 48 - 72 for frozen E. coli. At +2°C, the generation times of all the three strains were much high compared to +15°C or RT, Pseudomonas showing the lowest among the three (30 hours). The control (unfrozen) cells of the three strains possessed shortest generation time in first 24 hours at RT, 24 to 48 hours at +15°C and 72 - 96 hours at +2°C. In this respect, the behaviour of the unfrozen cells were very much similar to that of frozen cells. Only exception was the behaviour of E. coli at +15°C in which case the shortest generation times of the frozen and unfrozen cells differed by 24 hours.

At RT, the generation times of Pseudomonas, E. coli and Micrococcus were 1.6, 1.48 and 1.63 for frozen cells and 1.26, 1.34 and 1.49 for unfrozen cells. At +15°C, the shortest generation time of Pseudomonas, E. coli and Micrococcus were 2.58, 5.16 and 3.61 for frozen cells and 2.4, 2.72 and 2.83 for unfrozen cells. At +2°C, the corresponding values for frozen cells were 30, 37 and 72.2 and for unfrozen cells 26.6, 72 & 100. These results indicated that Pseudomonas, whether frozen or unfrozen, showed little difference in generation time and possessed the

greatest growth rate as indicated by lowest generation time at RT, +15°C and +2°C. A higher generation time was noted for frozen E. coli and Micrococcus at +15°C and +2°C than control. The ability to grow at +2°C is a characteristic of psychrotrophic strains, since many of the psychrotrophs possess minimum growth temperature at +2°C or below that (Eliott and Michener, 1964). Hence, the psychrotrophic nature of the Pseudomonas may be the reason for the identical growth rate.

An increase in the lag phase and generation time has been considered as the characteristic of injured cells, injured due to freezing (Straka and Stokes, 1959). Hence, the observed increase in lag or generation time of frozen cells pointed to the presence of injured cells. But this study also showed that injured cells alone is not the factor for increase in generation time, but the temperature of thawing is also important. This is because had it been due to injury alone, the lag and generation time should have been equal at all temperature for any individual frozen strain. Since the generation times were found to decrease considerably with lowered temperatures for each strain, it may be argued that temperature of thawing is crucial factor which governs growth of thawed cells.

There are only limited studies on the growth of individual bacterial cultures during thawing and most of them

deals with the behaviour of mixed cultures of bacterial populations and in substrates other than fish (Keruluck et al., 1961; Peterson et al., 1962a,b; Kraft and Ayres, 1966). This study deviates from those mentioned above in that it deals with behaviour of individual cultures in fish muscle. The work of Peterson et al. (1962a,b) reported that in mixed cultures, growth of E. coli, Staphylococcus aureus and Streptococcus fecalis in chicken at +15°C was greatly exceeded by Pseudomonas when the latter was present in small numbers and that the growth of Pseudomonas was about 9 times that of Staphylococcus aureus. This study supports the above finding and furtner show that the behaviour of Pseudomonas in individual culture is very much similar to that in mixed cultures. In this study, from the generation times, it was found that growth of Pseudomonas was approximately 2 times that of E. coli and Micrococcus at +15°C in the first 48 hours and 6 times that of E. coli and Micrococcus at +2°C in fish muscle.

4.4 Bacteriology of freezing of mackerel Rastrelliger kanagurta and prawn M. dobsoni

4.4.1 Changes in the bacterial flora of mackerel and prawn Metapenaeus dobsoni during preprocess-handling

4.4.1.1 Changes in the bacterial flora of mackerel Rastrelliger kanagurta

4.4.1.1.1 Quantitative aspects

Major changes occurring in the TPC of mackerel during preparation for freezing are illustrated by Tables 37 and 38. SWA and TGA were used as plating media with incubation at 5°C, room temperature (29±2°C) and 37°C.

At room temperature, the muscle of fresh mackerel registered a TPC ranging from 7.34×10^4/g to 1.15×10^4/g in SWA and 6.08×10^4/g to 9.47×10^5/g in TGA. The raw mackerel was immediately iced and brought to the laboratory within 1-2 hours.

The muscle of iced fish had a bacterial count ranging from 6.37×10^4/g to 9.92×10^4/g on TGA and 7.29×10^4/g to 1.17×10^6/g in SWA, the TPC being estimated within 3 hours after icing.

Washing the material with potable water resulted in considerable reduction in bacterial numbers. The percentage of reduction, due to washing as observed in SWA, ranged from 51.74 to 89.83 and the corresponding reduction in TGA was 73.87 to 90.98. This indicated that eventhough raw and iced fish showed a higher bacterial count on SWA than on TGA, the difference seemed less after washing. According to Shewan (1961), most of the bacteria adhering to slime and skin surface are washed away by water.

Table 37

Qualitative changes in the microbial flora of mackerel R. kanagurta during preprocess handling as on SWA (total plate count$\times 10^3$/g)

	Trial No.	Bacterial count* on SWA at 5°C	RT 29±2°C	37°C	Percentage increase/ decrease of the TPC at RT
Raw fish	1	83.5 (7.26)	1150.0	700.0	-
	2	3.43 (4.67)	73.4	62.2	
	3	9.08 (7.5)	121.0	109.0	
Iced fish (iced for 3 hours)	1	80.71 (6.86)	1175.0	721.0	2.17 (+)
	2	3.11 (4.26)	72.9	64.9	0.68(-)
	3	9.12 (7.06)	129.0	97.0	6.61 (+)
Washed (with potable water)	1	4.87 (4.16)	117.0	181.0	89.83 (-)
	2	0.22 (2.08)	10.1	9.98	86.15 (-)
	3	1.40 (2.4)	58.4	62.1	51.74 (-)
Glazed and packed	1	4.89 (4.14)	118.0	127.0	0.85 (+)
	2	0.18 (1.65)	10.9	11.3	7.92 (+)
	3	0.93 (1.56)	59.3	58.2	1.54 (+)

(+)Indicates an increase in bacterial number and (-) a decrease

*Average of 3 trials

Value in the parenthesis shows the percentage of psychrotrophs to the total count at RT

Table 38

Quantitative changes in the microbial flora of mackerel R. kanagurta during preprocess handling as on TGA (total plate count x 10^3/g)

	Trial No.	Bacterial count* on TGA at 5°C	RT (29±2°C)	37°C	Percentage increase/ decrease of the TPC at RT
Raw fish	1	64.70 (6.8)	94.7	910.0	-
	2	4.84 (7.9)	60.8	59.2	-
	3	2.18 (2.79)	78.0	71.0	-
Iced fish (iced for 3 hours)	1	62.90 (6.3)	99.2	997.0	4.75 (+)
	2	4.34 (6.8)	63.7	62.3	4.76 (+)
	3	2.39 (2.86)	83.4	81.7	15.00 (+)
Washed (with potable water)	1	3.61 (4.0)	90.0	90.0	90.98 (-)
	2	0.19 (2.46)	7.2	7.7	88.45 (-)
	3	0.54 (2.47)	21.8	20.61	73.87 (-)
Glazed and packed	1	3.58 (3.89)	92.0	92.0	1.1 (+)
	2	0.20 (2.73)	7.32	7.41	1.63 (+)
	3	0.51 (2.22)	22.98	23.12	5.41 (+)

(+) stands for an increase in bacterial count and (-) decrease

*Average of 3 trials
Value in the parenthesis shows the percentage of psychrotrophs to the total count at RT

The psychrotrophic count at 5°C of the raw material, which ranged from $2.18x10^3$/g to $6.47x10^4$/g in TGA came down to $5.1x10^2$/g to $3.58x10^3$/g for the final material ready for freezing. According to the observation of Gillespie and Macrae (1975), washing the tropical fishes with chlorinated water affected the Gram-negative psychrotrophs more than the Gram-positive mesophiles. Hence, the decrease observed here could be either due to the selective action of chlorinated water on psychrotrophs or due to effect of washing. Also, this points to the fact that washing does not add to the psychrotrophic flora as was stated by some workers (Shewan, 1971b, Lee and Pfeifer, 1977).

The quality of the raw material used in the study conformed to the quality standards proposed by Abeta (1983). It was also found to agree with the data reported for freshly landed fish (Zuberi and Quadri, 1980; Lakshmanan et al., 1985).

4.4.1.1.2 Qualitative aspects

The changes in the distribution of major microbial groups on mackerel during preparation for freezing was studied (Table 39). Only the colonies developing on TGA were identified. Since the major changes during preprocess handling and subsequent stages were thought to be due to contamination, it was envisaged that TGA could illustrate the results more precisely.

Table 39

*Changes in the distribution of major microbial groups in skin and muscle of mackerel R. kanagurta during preprocess handling as on TGA

Bacterial species	Raw			Iced			Washed			Glazed and packed		
	5°	RT	37°	5°	RT	37°	5°	RT	37°	5°	RT	37°
	**											
Pseudomonas	26	23	16	28	26	17	28	28	27	26	22	20
Moraxella	10	11	8	12	13	8	11	8	9	7	6	6
Acinetobacter	11	12	11	9	9	12	9	4	5	9	6	5
Vibrio	28	31	38	25	28	35	19	22	24	18	20	24
Flavobacterium/ cytophaga	9	9	10	7	8	8	6	6	5	7	6	5
Alcaligenes	4	2	1	2	3	1	3	3	-	2	-	1
Micrococcus	5	6	7	5	8	8	22	27	29	28	34	33
Bacillus	2	2	3	1	2	1	1	2	-	2	-	1
Arthrobacter	2	4	4	4	3	3	1	0	-	1	6	5
Not identified	3	0	2	7	0	7	0	0	1	0	0	0
Total no. of isolates	93	92	90	61	84	73	78	72	70	81	63	84

* Colonies isolated from TGA media

** Expressed as percentage of the total

The composition of the microbial flora of raw fish at RT was characterized by the predominance of Vibrio (31%) over others. This was followed by Pseudomonas (23%), Acinetobacter (12%), Moraxella (11%) and Flavobacterium (9%). The bacterial flora of the iced fish was very much similar to that of raw fish except a slight increase in the number of Pseudomonas and decrease in Vibrio spp.

Washing caused a decrease in the number of Moraxella, Vibrio, Acinetobacter and Flavobacterium species and increase in the proportion of Gram-positive Micrococci. Pseudomonas species were less affected. Since the contamination from water is negligible, the increase in percentage of Gram-positive cocci should be due to human handling.

4.4.1.2 Changes in the bacterial flora of prawn Metapenaeus dobsoni

4.4.1.2.1 Quantitative changes

The changes in the total plate count of prawn M. dobsoni during preprocess handling for freezing are illustrated in Tables 40 and 41. The total bacterial count was determined in two media SWA and TGA at three temperatures of incubation viz. 5°C, room temperature (29±2°C) and 37°C.

The TPC of the raw material ranged from 7.87×10^5/g at RT to 3.13×10^6/g on SWA.. On TGA, the bacterial count

Table 40

Quantitative changes in the microbial flora of prawn M. dobsoni during preprocess handling as on SWA

*(Total plate count x 10^3/g)

	No. of trials	Bacterial count on SWA at 5°	RT 29±2°C	37°C	Increase/ decrease (%)
Raw	1	104 (4.92)	2110	1370	-
	2	200 (6.38)	3130	2860	
	3	542 (5.33)	787	627	
Beheaded	1	411 (4.11)	996	984	52.68 (-)
	2	78 (6.56)	1189	958	62.01 (-)
	3	34 '6.77)	502	311	36.21 (-)
Iced	1	39 (4.41)	883	863	11.40 (-)
	2	82 (6.72)	1220	1280	2.60 (+)
	3	27 (4.72)	572	438	14.30 (+)
Peeled and deveined	1	31 (3.88)	797	737	9.73 (-)
	2	71 (5.13)	1383	1367	11.78 (+)
	3	22 (3.20)	687	694	20.10 (+)
Washed and packed	1	13 (1.07)	121	125	80.71 (-)
	2	13 (1.94)	684	691	50.54 (-)
	3	32 (1.52)	211	342	69.28 (-)

(+) stands for increase in bacterial count and

(-) for a decrease

*Average of 3 plates

The value in the parenthesis shows the percentage of psychrotrophs to the total count at RT

Table 41

Quantitative changes in the microbial flora of prawn *M. dobsoni* during preprocess handling as on TGA

*(total plate count x 10^3/g)

	No. of trials	Bacterial count on TGA at 5°C	RT 29±2°C	37°C	Increase/ decrease (%)
Raw	1	13.7 (2.19)	624	383	-
	2	58.0 (5.37)	1080	1050	
	3	20.0 (4.16)	480	472	
Beheaded	1	91.0 (2.76)	325	315	48.02 (-)
	2	36.0 (5.78)	623	608	42.30 (-)
	3	821.0 (3.85)	213	208	55.63 (-)
Iced	1	803.0 (2.58)	310	320	4.62 (-)
	2	341.0 (5.35)	635	636	1.92 (+)
	3	847.0 (3.71)	228	201	7.00 (+)
Peeled and deveined	1	49.0 (0.95)	513	527	65.48 (+)
	2	31.0 (3.30)	940	959	32.44 (+)
	3	10.8 (3.23)	334	359	46.49 (+)
Washed and packed	1	0.128 (0.096)	133	144	74.07 (-)
	2	26.0 (1.03)	251	259	73.22 (-)
	3	0.88 (0.81)	108	123	67.66 (-)

(+) stands for increase in bacterial count and

(-) for decrease

*Average of 3 plates

The value in the parenthesis shows the percentage of psychrotrophs to the total count

fell within the range of 4.8×10^5/g to 1.08×10^6/g at RT.

Beheading the prawn lowered the bacterial count at RT on SWA from 7.87×10^5/g to 5.02×10^5/g. There was a decreas in TPC due to beheading and the percentage reduction varied from 36.21 to 62.01 on the basis of count at RT. In TGA, the reduction varied from 42.3% to 55.63%.

Icing made no significant change as was seen from the data. In SWA, at RT, there was increase of 2.6% and 14.3% in two cases and in one instance there was a decrease by 11.4%. According to Castell et al. (1954), Georgala (1957b) and Iyer and Choudhuri (1966), icing could introduce bacteria causing higher bacterial count in sample. Eventhough an increase was noted in this study, the increase was negligible. The exposure of the sample to ice was very brief, ranging only a few hours and that may be the reason for this type of behaviour.

Peeling and deveining resulted in increase in bacterial count. The increase as percentage on TGA at RT ranged from 32.44 to 65.48. On the otherhand, there was only slight increase in two instances on SWA and in one case even a decrease was noted. This clearly shows that the observed increase in TPC in TGA is due to contamination.

Washing had an effect in decreasing the bacterial load considerably. In this respect, the trend here was

similar to that observed for mackerel. The decrease, as percentage, was 50.54 to 80.71 on SWA at RT and 67.77 to 74.07 on TGA.

The TPC of the raw material reported in this study agrees well with that reported earlier. Shewan (1971b) reported TPC of the shrimp to be in the range of 10^4-10^6/g, 10^5/g being very common. Similar values were also reported by Harrison & Lee (1969), Hobbs et al. (1971), Baer et al. (1976) and Lee and Pfeifer (1977) for shrimp from temperate waters.

As in the case of mackerel, the psychrotrophic count at 5°C in both SWA and TGA showed downward trend. In SWA, there was a decrease in their percentage at 5°C from 4.92-6.38 to 1.07-1.94 and in TGA, the corresponding values were 2.9% - 5.4% to 0.96% - 1.05%. The same reason that had been suggested in the case of mackerel could be applied here also.

The head portion of the prawn constituted 40% of the body weight, while it carried 75% of the bacteria (Novak, 1973). Hence, the decrease observed in bacterial count due to beheading is understandable. Also, the reduction in bacterial count observed according to present study agrees well with that reported by Williams et al. (1952) and Novak (1973). However, other workers stated that no

difference existed between whole and headless shrimp (Koburger et al. 1973; Cann, 1977; Alvarez and Koburger, 1978).

The effect of washing and picking/peeling on the bacterial count of shrimp was studied by Harrison and Lee (1969) and Lee and Pfeifer (1977). Other shellfishes like crab was studied by Loharan and Lopez (1970), Philips and Peeler (1972), Lee and Pfeifer (1975) and Ray et al. (1976). The values reported here are substantiated by the studies mentioned above.

It is clear from this study that washing and peeling are the two steps which decides the bacterial count of the raw material. Still, the overall reduction in the bacterial count of the finished material is less than one order of raw material. As stated by Hobbs et al. (1971), apart from a slight contamination in the peeling stage and partial removal of the commensal bacteria, the preprocess-handling does not significantly affect the raw material.

4.4.1.2.2 Qualitative aspects

The qualitative changes in the microbial flora of prawn M. dobsoni during preprocess handling are given in Table 42. Only the bacterial colonies developing on TGA were identified.

Table 42

*Changes in the distribution of major microbial groups in the muscle of prawn M. dobsoni during preprocess-handling as on TGA

Bacterial species	Raw prawn			Beheaded prawn			Iced prawn			Peeled & deveined			Washed and ready for freezing: Whole			Headless			PD		
	Temperature of incubation °C																				
	5	RT	37	5	RT	37	5	RT	37	5	RT	37	5	RT	37	5	RT	37	5	RT	37
Pseudomonas	22**	17	12	20	18	14	24	24	23	11	11	7	21	20	20	23	23	24	6	6	5
Moraxella	22	20	18	21	18	17	20	21	19	8	12	11	17	17	16	18	13	15	10	10	11
Acineto-bacter	18	19	20	19	21	20	19	18	18	12	11	12	16	16	17	16	17	14	12	12	12
Vibrio	15	18	22	16	18	23	14	15	15	12	12	14	12	14	14	14	15	15	13	14	13
Flavabac-terium/ cytophaga	10	9	10	8	9	10	18	6	10	6	4	4	8	6	3	3	4	4	6	4	4
Enteroba-cteriaceae	0	0	0	0	0	0	0	0	1	0	2	2	0	1	1	0	0	1	0	0	2
Micrococcaceae	5	6	7	3	5	6	3	4	4	39	40	40	19	20	25	20	23	24	46	47	47
Bacillus	0	1	1	2	1	2	1	2	1	4	2	3	3	2	2	2	1	1	3	3	2
Arthro-bacter	8	10	10	11	10	8	1	10	9	8	6	7	4	4	2	4	4	2	4	4	4
Total no. of isolates	72	83	79	81	88	70	88	76	81	70	68	82	73	78	77	85	83	83	68	80	80

*Colonies isolated from TGA media; ** Expressed as percentage of total isolates

The most striking feature of prawn in preprocess-handling was the increase in Gram-positives, mainly Micrococcus spp. These bacteria which constituted 6% of raw prawn at incubation temperature of RT rose to 47% in the peeled material ready for freezing. Harrison and Lee (1969) attributed this increase in the Gram-positive flora to handling by human hands. This trend was noted by Lee and Pfeifer (1975) in crab processing and by Lee and Pfeifer (1977) in shrimp processing.

The Moraxella-Acinetobacter group were the most predominating microbial genera encountered in the raw shrimp, at RT. Their number remained stationary after beheading also. But the subsequent steps of icing, peeling and deveining and washing caused a decrease in the number of these bacteria. Similar trend was noted in the case of Vibrio spp. and Flavobacterium spp.

But the Pseudomonas spp., which was almost equal to Vibrio in population density, remained like that after beheading. Icing caused an increase of this group from 18 to 24%. Their percentage in whole and beheaded prawn increased after washing. In peeled and deveined prawn, a drastic fall occurred.

Similar works reported for shrimp from cold waters are that of Harrison and Lee (1969) and Lee and Pfeifer (1977)

on Pacific shrimp, Pandalus jordani and in crab, Cancer magister (Lee and Pfeifer, 1975). But the values are not quite comparable to the present study, since all these processes involved cooking/blanching and brining. Work reported in India on similar line is not as extensive and are restricted to changes in TPC and pathogenic/indicator bacterial count. Harrison and Lee (1969) noted a slight decrease in Pseudomonas and Flavobacterium species during processing. Moraxella-Acinetobacter group, which was present in increased proportion in the cooked material, reduced during further processing. The Gram-positive cocci increased from 7.1 to 76%. But according to Lee and Pfeifer (1977), Pseudomonas increased from 17 to 44% in Plant A, while in Plant B it remained same. Also, Moraxella was totally eliminated while Acinetobacter and Flavobacterium increased. During processing of crab Cancer magister, Lee and Pfeifer (1975) noted a decrease of Pseudomonas from 18 to 8% and of Moraxella from 45 to 27%. Acinetobacter and Flavobacterium, which initially constituted 15% and 6% remained at the same level (18% and 8% respectively) till the end. Micrococcus increased from 2 to 19% (Lee and Pfeifer, 1975).

A high preponderance of Micrococcus in the flora of prawn during processing was observed in the present study. But this group was found to constitute negligible

portion in the processed shrimp as per the studies reported by Lee and Pfeifer (1977). The reason could be that while hand peeling was followed in the present study, machine peeling was adopted by Lee and Pfeifer (1977). The data presented by Harrison and Lee (1969), which followed hand peeling, showed similar increase in Gram-positive cocci mainly, Micrococcus during processing.

4.4.2 Changes in the bacterial flora during freezing and frozen storage of mackerel/prawn

4.4.2.1 Changes in the bacterial flora during freezing and frozen storage of mackerel R. Kanagurta

4.4.2.1.1 Quantitative aspects

Tables 43 and 44 shows the effect of freezing at -40°C and subsequent storage at -20±2°C on the bacteria present in skin and muscle and in intestine with contents of mackerel (R. kanagurta). The data relates to the recovery of bacteria on two different media viz. SWA and TGA and incubated at three temperatures namely, 5°C, RT (29±2°C) and 37°C. The plates incubated at 5°C were subsequently incubated for one day at RT and this count represented 5/30 count.

The total bacterial count of skin and muscle of mackerel (R. kanagurta), just before freezing, ranged from 2.4×10^4/g to 8.5×10^5/g in SWA and 9.8×10^3/g to 6.4×10^5/g in TGA.

Table 43

[1]Changes in the total bacterial count of skin and muscle of mackerel *R. kanagurta* during block freezing at -40°C and storage -20±2°C

*(total plate count x 10^3/g)

Period of frozen storage (months)	Count on SWA				Count on TGA			
	5°C	5/30°C	RT (29±2°C)	37°	5°	5/30°C	RT (29±2°C)	37°C
Initial	4.80	108.00	118.00	127.00	3.58	81.00	92.00	92.00
0	3.80	13.00	17.00	18.30	3.37	8.43	16.30	10.70
1	3.80	12.00	12.50	8.90	7.27	7.26	10.50	9.10
2	7.20	9.20	10.40	6.12	9.80	10.00	19.30	13.00
4	2.00	3.50	3.50	2.70	6.70	8.30	9.20	8.90
6	4.90	5.10	5.30	4.80	4.80	4.90	5.80	5.60
10	0.79	0.71	0.88	0.78	0.52	0.58	0.61	0.52
12	0.65	0.66	0.65	0.76	0.59	0.61	0.61	0.61

*Average count of triplicate plates in a typical series.

1 3 series of freezing and frozen storage studies were undertaken (i) May, 1982 to Feb. 1983 (ii) August 1982 to July, 1983 and (iii) May 1983 to April 1984. Comparative results were obtained. The data presented here represents results of a typical study.

Table 44

[1]Changes in the TPC of the intestine with contents of mackerel *R. kanagurta* during block freezing at -40°C and storage at -20±2°C *(total plate count x 10^3/g)

Period of frozen storage (months)	Count on SWA				Count on TGA			
	+5°C	5/30°C	RT (29±2°C)	37°C	+5°C	5/30°C	RT (29±2°C)	37°C
Initial	21.00	24.0	6710.00	5739.0	4.26	76.70	837.00	911.0
0	0.61	7.61	9.23	8.90	0.347	5.83	8.18	8.2
1	0.29	3.09	6.60	4.98	0.710	2.10	3.15	3.0
2	1.66	2.60	5.36	3.71	1.240	1.28	1.03	1.2
4	1.91	4.00	4.78	5.88	1.500	1.89	3.81	3.2
6	1.80	2.00	2.64	3.89	1.100	1.10	2.11	2.4
10	3.30	3.90	3.00	3.10	-	-	-	-
12	-	-	-	-	-	-	-	-

*Average count of triplicate plates in a typical series

1 3 series of freezing and frozen storage studies were undertaken (i) May, 1982 to Feb. 1983 (ii) August, 1982 to July, 1983 (iii) May, 1983, to April, 1984 comparative results were obtained. The data presented here represents results of a typical study.

On freezing at -40°C for 6 hours, the counts fell to $1.7x10^4$/g to $5.2x10^5$/g in SWA and $9.8x10^3$/g to $1.1x10^5$/g in TGA. After frozen storage at -20±2°C for 4 months, the bacterial counts came down to $2.3x10^3$/g to $1.1x10^4$/g in SWA and $2.9x10^3$/g to $9.5x10^3$/g in TGA. Typical results from this study are presented in Table 43. The changes occurring in the TPC of intestine is given in Table 44.

For raw material as well as the material just before freezing, SWA gave a higher count than TGA, at all temperatures. This was true for skin and muscle as well as intestinal contents. However, this difference in the recovery of bacteria on the two media almost disappeared after freezing and frozen storage of fish. The possible explanation could be that bacteria, which specifically required sea water for growth, were replaced by a more homogeneous flora which could grow in both SWA and TGA.

Among the three temperatures of incubation viz. 5°C, RT and 37°C, the room temperature consistently gave higher recoveries, than the other two temperatures during freezing and the entire period of frozen storage. The effect of temperature was more conspicuous on skin and muscle samples in SWA, where throughout the storage period the highest count was observed at RT. Intestinal contents, on the otherhand, showed a slight increase in the count at 37°C.

The plates incubated at 5°C on subsequent incubation at RT gave an increased colony count as seen in Tables 43 and 44. The ratio of count at 5°C to that 5/30 is given in Table 45. The value 5/5/30 increased in skin with muscle and intestine in both media. For skin with muscle and intestine the values before freezing was 0.0432 and 0.08 which increased to 0.90 and 0.80 after 10 months of storage. This indicated that the difference between the two counts lessened with frozen storage. One reason for this increase in count could be the development of micro-colonies of psychrotrophs. These colonies failed detection on incubation at 5°C for 21 days, but grew to visible size on subsequent incubation at 30°C. It can also be due to the growth of mesophiles which remained dormant, but viable at low temperature. The former possibility has been pointed out by Fabri et al. (1982)

The total bacterial count of fish before freezing was lower at 37°C than at room temperature (Table 46). The ratio of the count at RT to that at 37°C was greater than unity and remained like that up to 10 months. This could be possible only if there is a uniform fall in count at RT and 37°C. Conversely, bacteria growing at RT and 37°C were affected at equal level by exposure to low temperature that may occur during freezing and frozen storage.

Table 45

Changes in the ratio of counts 5/5/30 in SWA and TGA for mackerel R. kanagurta during freezing and frozen storage

Period of frozen storage (months)	Skin and Muscle		Intestine	
	SWA	TGA	SWA	TGA
Initial	0.0432*	0.044	0.080	0.050
0	0.2920	0.399	0.080	0.050
1	0.3250	0.956	0.095	0.259
2	0.7820	0.980	0.638	0.336
4	0.5710	0.810	0.477	0.643
6	0.8300	0.979	0.900	0.793
10	0.9010	0.896	0.800	1.000
12	0.9750	0.975	-	0.960

*Based on results given in Tables 43 and 44.

Table 46

Changes in the ratio of counts at RT to that at 37°C in SWA and TGA for mackerel R. kanagurta during freezing and frozen storage

Period of frozen storage (months)	Skin and Muscle		Intestine	
	SWA	TGA	SWA	TGA
Initial	0.92*	1.00	1.17	0.92
0	0.93	1.52	1.03	0.99
1	1.40	1.15	1.32	1.05
2	1.69	1.48	1.44	0.86
4	1.30	1.03	0.81	1.19
6	1.10	1.04	0.79	0.88
10	1.01	1.17	0.76	0.86
12	0.86	1.18	-	-

*Based on results given in Tables 43 and 44

The total colony count at 5°C gives an idea of the prevalance of the psychrotrophic population in the fish before and after freezing and on frozen storage. The percentage of psychrotrophs in the total bacterial count at room temperature is given in Table 47. As seen from the table the percentage of psychrotrophs which initially constituted a minor fraction of the total population, went on increasing and constituted 88.86% of the population in skin and muscle (SWA) after 10 months. The entire population was psychrotrophic after storage for one year.

Freezing caused significant reduction in the bacterial count (Table 48). The percentage reduction in the total bacterial count in the case of block frozen mackerel in series I and II were respectively 82.60 and 74.25. The corresponding reduction in the case of individually quick frozen (IQF) mackerel was found to be 81.4 and 83.6 respectively. This showed that there was practically no difference in the reduction of bacteria by the two methods.

Table 47

Changes in the percentage of psychrotrophs on SWA and TGA in mackerel R. kanagurta during freezing and frozen storage

Period of frozen storage (months)	Intestine		Skin and Muscle	
	SWA	TGA	SWA	TGA
Initial	0.31*	0.51	4.14	3.89
0	0.62	4.24	22.30	20.67
1	4.35	22.40	31.20	69.20
2	63.80	80.00	69.20	50.77
4	40.04	39.37	57.14	7.28
6	68.18	52.13	92.40	82.70
10	73.17	58.04	88.60	85.25
12	75.30	66.43	100.00	99.17

*Based on results given in Tables 43 and 44

Table 48

*Percentage reduction in the total bacterial count at intervals during freezing and frozen storage of IQF and Block frozen mackerel

Period of frozen storage (months)	Series I		Series II	
	IQF	Block	IQF	Block
Initial	-	-	-	-
0	81.40	82.29	83.6	74.25
1	16.31	35.59	-	24.86
2	16.31	-	59.4	-
4	29.89	52.34	-	59.69
6	27.40+	37.00+	78.50+	8.14+
10	17.40	89.40	-	24.00
12	3.20	-	17.30	4.53

*Based on TPC on TGA at RT
+Count after storage for 5 months

During storage at -20±2°C for period up to one year, the reduction was less significant for both IQF and block frozen mackerel (Table 49). The reduction during storage period of one month for block frozen mackerel was only 35.59% in series I and 24.86% in series II. The values were found to be quite variable during frozen storage and varied from 3.2% to 29.89% for IQF and 35.59% to 89.4% for block. In certain instance even an increase in count was noted. This could be attributed to the disintegration of clumped bacteral cells due to freezing. Such phenomenon had been reported for pure cultures by some authors (Postgate and Hunter, 1961). Borgstrom (1955) pointed out that in the initial stages of freezing and frozen storage when the bacterial count is decreasing rather quickly there is a definite relationship between death rate and viable bacterial count, but during later stages bacterial count decreases less rapidly and no relationship exists.

Eventhough the decrease in bacterial count due to freezing had been investigated previously, the data could not be compared in toto to the present study. This is because of the variations in freezing conditions storage etc. Still, a comparision can reveal some valid information.

Kiser and Beckwith (1942), studying the effect of frozen storage at -28°C for 10 days on mackerel, found that the muscle exhibited a decrease in count of 43.3%,

while intestine showed a striking reduction of up to 97.9%. Pivnick (1949) also showed that freezing the fish muscle for 24 hours caused a decrease in bacterial population of 40-60%. Shewan (1961) reported 60-90% reduction in bacterial count during freezing and frozen storage of north sea fish. Ocean perch stored at -15°C after freezing showed 17% reduction in bacterial count after one month (Lee et al., 1967). After freezing at -40°C and storage at -15°C for 2½ months, 60% reduction was noted in the initial bacterial population for mackerel (Rastrelliger kanagurta), 60-70% for white pomfret (Stromateus cinereus) and 70% for Surmai (Cybium commersonii) (Jadhav and Magar, 1970). Ehira and Fuji (1980) reported 50% reduction for sardine (Sardinella melanosticta), frozen and stored at -30°C for 30 to 50 days. The values reported for Indian mackerel in the present study agree with these earlier reports.

The maximum reduction in bacterial count was observed in the early period of frozen storage after which the reduction was more or less gradual. This trend was noted by Jadhav and Magar (1970). Similar trend was noted in the behaviour of pure cultures during freezing and frozen storage.

Changes in the chemical indices during freezing and frozen storage are given in Figures 32.1 to 32.4 and

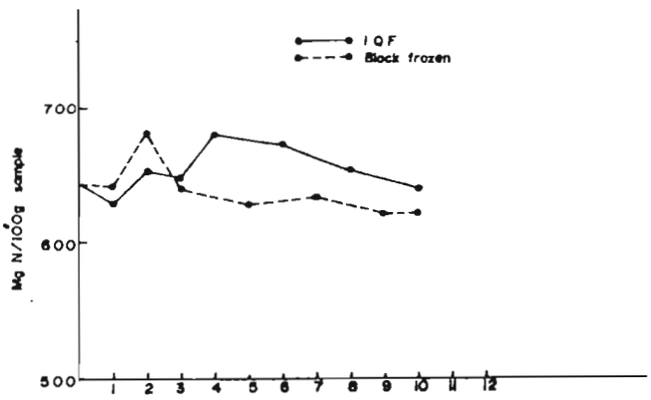

Fig 32.1
CHANGES IN NON-PROTEIN NITROGEN CONTENT OF MACKEREL R.KANAGURTA AFTER FREEZING AT -40°C AND STORAGE AT -20 ± 2°C

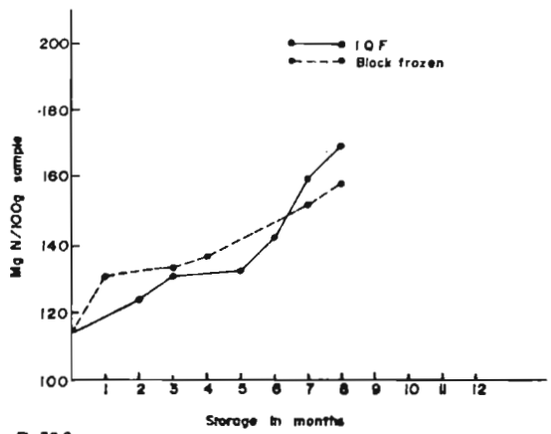

Fig.32.2.
CHANGES IN α-AMINO NITROGEN CONTENT OF MACKEREL R.KANAGURTA AFTER FREEZING AT -40°C AND STORAGE AT -20 ± 2°C

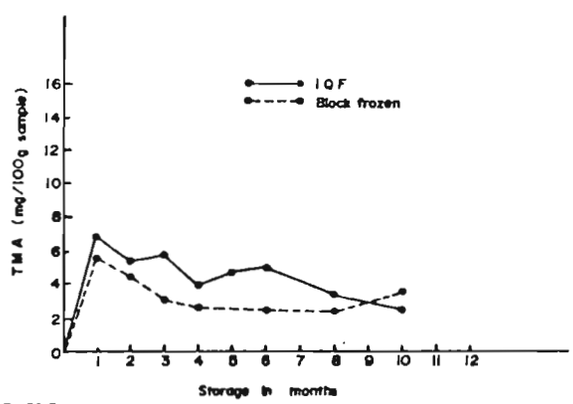

Fig 32.3.
CHANGES IN TRIMETHYLAMINE CONTENT OF MACKEREL R.KANAGURTA AFTER FREEZING AT -40°C AND STORAGE AT -20 ± 2°C

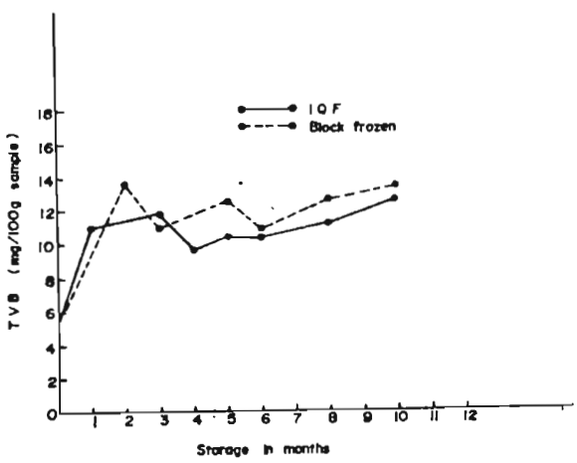

Fig. 32.4.
CHANGES IN TOTAL VOLATILE BASES OF MACKEREL R. KANAGURTA AFTER FREEZING AT -40°C AND STORAGE AT -20 ± 2°C

organoleptic scoring in Table 48. After 7 months, the fish samples were organoleptically and chemically in a poor condition. Still, the bacterial count was maintained low and this continued until the end of storage period when the fish was totally unacceptable. This points to poor correlation of bacterial count with the quality indices of frozen fish and prawn during freezing and frozen storage. Similar observation has been reported by Pillai *et al.* (1965).

4.4.2.1.2 Qualitative aspects

The changes in the different bacterial groups in mackerel (*R. kanagurta*) during freezing at -40°C and storage, at -20±2°C for period up to one year are presented with regard to skin and muscle (Table 50) and intestine with contents (Table 51). The changes occurring to bacteria after recovery on TGA at three different temperatures of incubation have been presented.

Freezing as well as frozen storage caused distinct changes in the bacterial flora of mackerel. The most striking feature was the replacement of the native Gram-negative flora by restricted species of Gram-positives. The Gram-positive flora, which constituted 34% of the flora just before freezing, rose to 58% of the total population at RT at the end of one year storage period. In intestines, the percentage of Gram-positives rose from 21% to 77%.

Table 49

Changes in the organoleptic characteristics of mackerel R. kanagurta after freezing at -40°C and frozen storage at -20±2°C

Period of storage (weeks)	IQF				Block frozen			
	Batch I	Total score	Batch II	Total score	Batch I	Total score	Batch II	Total score
0	Excellent	9	Excellent	9	Excellent	9	Excellent	9
4	Good	8	Good	8	Good	8	Good	8
8	Good slightly tough	8	Good slightly tough	7	Good	8	Good	8
12	Fair	6	Good to fair slight toughness in texture	6	Good slight toughness	7	Good	7
16	Slightly rancid	5	Fair slightly tough rancid	5	Good to Fair slight rancid and tough	6	Good	7
24	Rancid very tough	4	off odour Off odour	4	Fair, mild rancidity and tough	5	Fair, tough rancid	6
32	Poor, very tough very rancid	3	Poor, very rancid, very	3	Fair, rancid very tough	4	Fair, rancid, tough	4

Table 50

Changes in the distribution of major microbial groups in skin and muscle of mackerel R. kanagurta during freezing at -40°C and storage -20±2°C

Period of frozen storage (months)	Temperature of incubation °C	Pseudomonas	Acinetobacter	Moraxella	Vibrio	Flavobacterium/cytophaga	Micrococcus	Bacillus	Arthrobacter	Others	Total isolates
Initial	5	26*	9	7	8	6	28	0	6	0	91
	RT	20	6	6	21	6	34	0	6	5	80
	37	20	5	6	24	5	33	1	6	5	80
0	5	17	4	7	6	4	37	0	5	0	94
	RT	25	7	8	10	4	40	0	6	0	81
	37	24	7	9	11	4	40	0	5	0	71
1	5	21	19	12	0	2	42	0	4	0	78
	RT	33	6	11	0	6	44	0	0	0	75
	37	32	7	10	0	5	45	1	1	1	86
2	5	23	20	8	0	2	43	0	3	1	92
	RT	34	6	5	0	5	45	0	5	0	84
	37	29	5	5	0	2	50	2	6	1	93
6	5	28	19	11	0	2	40	0	0	0	87
	RT	15	18	14	0	3	49	0	0	0	77
	37	15	9	14	0	0	55	2	5	0	96
10	5	15	10	27	0	0	48	0	0	0	75
	RT	11	14	15	0	2	57	0	1	0	77
	37	10	8	14	0	0	62	2	4	0	82
12	5	18	9	27	0	0	42	0	0	4	82
	RT	12	16	13	0	0	58	0	1	0	75
	37	12	8	11	0	0	66	0	3	0	86

*Expressed as percentage of the total

Table 51

Changes in the distribution of major microbial groups in the intestine of mackerel R. kanagurta during freezing at -40°C and storage at -20±2°C

Period of frozen storage (months)	Temperature of incubation °C	Pseudomonas	Acinetobacter	Moraxella	Vibrio	Flavobacterium/ cytophaga	Micrococcus	Bacillus	Arthrobacter	Others	Total isolates
Initial	5	17*	28	22	12	1	10	4	6	0	77
	RT	17	10	19	25	6	6	3	12	1	91
	37	14	15	16	24	0	7	12	12	-	73
0	5	18	26	20	0	0	22	11	3	0	80
	RT	13	16	15	6	0	22	18	10	0	90
	37	14	10	14	2	0	20	23	17	0	89
1	5	12	33	8	0	0	33	10	4	0	91
	RT	12	9	10	0	0	30	25	14	0	88
	37	14	14	15	0	0	25	18	13	0	94
2	5	16	30	11	0	0	30	11	2	0	91
	RT	14	9	3	0	0	29	27	15	3	87
	37	11	12	12	0	0	39	14	12	0	85
6	5	18	21	13	0	0	34	11	3	0	83
	RT	14	14	8	0	0	36	24	4	0	81
	37	11	15	9	0	0	44	12	9	0	79
10	5	22	20	12	0	0	34	9	3	0	85
	RT	12	10	8	0	0	38	28	4	0	84
	37	11	10	8	0	0	48	12	11	0	80
12	5	12	24	14	0	0	36	12	1	0	71
	RT	9	8	6	0	0	50	24	3	0	88
	37	10	11	7	0	0	48	10	14	0	82

*Expressed as percentage of the total

The incubation temperature showed some influence on the recovery of certain bacterial groups during analysis eventhough it was not so evident as in the case of fresh fish. Gram-negative Pseudomonas and Moraxella appeared in greater numbers on plates incubated at 5°C especially in the beginning of the frozen storage. Similarly, the number of Micrococcus was lesser at 5°C than 30°C which in turn was lesser than that at 37°C. This trend was more clear towards the later stages of frozen storage. The recovery of Micrococcus at 5°C, RT and 37°C after storage for 10 months were respectively 48%, 57% and 62%. This can probably be a reflection of the population effect since in the beginning the Gram-negatives were the predominant flora and towards the end the Gram-positives predominated.

In skin and muscle, rapid destruction of Vibrio species occurred during freezing and they disappeared completely after one month storage. At RT, the Vibrio spp. were 21% of the total which came down to 10% after freezing. None could be detected after one month storage of mackerel at -20 ± 2°C. While the percentage of Vibrio registered a steep decline, the Micrococcus showed gradual increase in their proportion i.e. from 34% before freezing to 58% at the end of frozen storage. The Pseudomonas showed a gradual decrease, while Acinetobacter and Moraxella showed increase

in relative numbers with the progress of frozen storage.

The behaviour of Vibrio spp. in intestine was similar to that of skin and muscle during freezing and subsequent frozen storage. The percentage of Arthrobacter was considerably high in intestine with contents compared to skin with muscle and their number decreased from 12% before freezing to 3% at the end of frozen storage. Contrary to this, the Bacillus group showed an increase from 3 to 24%.

Kiser and Beckwith (1942) found Micrococcus and Achromobacter to be the most resistant in frozen mackerel. In frozen ocean perch, Lee et al (1969) observed a high incidence of Pseudomonas (26.9%) followed by Achromobacter (22%) and Flavobacterium (12.1%). The Gram-positives formed 24.2% of the flora and were composed of Coryneforms, Bacillus and Lactobacillus.

The qualitative nature of the flora of the frozen fishes like mackerel, white pomfret and surmai from Bombay coast were reported to carry higher numbers of Gram-positives, chiefly Bacillus and Micrococcus in addition to a lesser proportion of Gram-negatives, mainly Achromobacter. (Jadhav and Magar, 1970). The present data agrees more with that of Lee et al. (1969). The higher incidence of Coryneforms, reported by Lee et al. (1969)

for ocean perch, was observed in the case of intestine of mackerel in this study.

The Vibrio spp. were the most affected by freezing and frozen storage. This observation was supported here by the studies with pure cultures of Vibrio. But in fish muscle media the Vibrios were found to survive for less than 3 months at -20±2°C, whereas in fish/prawn they were absent on storage at -20±2°C for one month. This may be due to the fact that the proportion of Vibrio in fish after freezing constituted only a small fraction of the total population so that they escaped detection particularly, in the dilutions used for total plate count.

The frozen samples were also monitored for the presence of pathogenic/indicator bacteria. Pathogens like Salmonella, Vibrio parahaemolyticus were absent throughout the study. Coagulase positive Staphylococci, coliforms, E. coli and fecal streptococci were low in number in skin and muscle and in most cases less than 100.

Thus, eventhough the survival of these pathogenic/indicator bacteria is considerable in fish muscle media, the risk imposed by them in fish is quite insignificant because of their low incidence in raw material. But in cases where the initial contamination is high in raw material, the frozen product is likely to endanger the consumer.

4.4.2.2 Changes in the bacterial flora of prawn *Metapenaeus dobsoni* during freezing and frozen storage

4.4.2.2.1 Quantitative studies

Changes in the total bacterial count of the muscle of prawn *M. dobsoni* frozen as whole, headless and PD at -40°C in contact plate freezer for 6 hours and stored at -20±2°C up to one year are presented in Tables 52 and 53. The effect of three temperatures of incubation and two media are also discussed. The plates incubated at low temperature (5°C) were subsequently kept at RT for one day and this represented 5/30 count.

In TGA, the total bacterial count of the muscle of the prawn *M. dobsoni* just before freezing ranged from 5.33×10^5/g - 1.82×10^6/g in whole prawn, 2.92×10^5/g - 5.32×10^5/g in headless and 1.21×10^5/g - 6.84×10^6/g in peeled and deveined (PD). However, freezing at -40°C for six hour decreased the total bacterial count of muscle of prawn to 3.1×10^4/g - 5.27×10^5/g in prawn frozen as whole, 2.3×10^4/g - 4.7×10^4/g in headless and 9.03×10^3/g to 3.2×10^5/g in PD. After storage for one month at -20±2°C in deep freezer, the bacterial count of the three types of prawn were respectively 5.3×10^3/g - 9.8×10^3/g, 2.4×10^3/g - 6.4×10^3/g and 4.03×10^3/g - 6.7×10^3/g. The total bacterial counts of prawn frozen as whole, headless

Table 52

Changes in the TPC of the muscle of prawn M. dobsoni during freezing and storage at -20±2°C as on SWA (total plate count x 10^3/g)

Period of storage in months	Bacterial count on SWA											
	Whole prawn				Headless				Peeled and deveined			
	Temperature of incubation °C											
	5	5/30	RT	37	5	5/30	RT	37	5	5/30	RT	37
Initial	23.9	187.00	533.00	533.00	21.00	400.00	415.00	410.00	6.5	300.00	348.00	345.00
0	11.5	63.00	92.00	63.00	0.98	21.80	33.10	21.70	9.9	94.00	131.00	106.00
1	1.2	5.70	12.30	9.45	2.90	6.10	5.80	6.20	22.0	52.00	81.00	78.00
2	0.32	0.82	1.25	1.23	2.79	3.30	3.60	3.50	18.0	42.00	58.00	57.00
6	0.37	0.39	0.38	0.39	0.91	0.99	0.98	0.98	8.0	10.00	10.00	16.00
10	0.10	0.12	0.12	0.13	0.48	0.49	0.49	0.49	4.1	4.20	4.40	4.30
12	0.10	0.11	0.17	0.19	0.29	0.36	0.36	0.37	3.2	3.90	3.90	3.90

Note: 3 series of experiments were undertaken during the period August, 1983 to April, 1985. comparable results were obtained. Typical results are presented here.

Table 53

Changes in the TPC of the muscle of prawn M. dobsoni during freezing at -40°C and frozen storage at -20±2°C as on TGA (total plate count x 10^3/g)

Period of storage (months)	Bacterial count on TGA											
	Whole prawn				Headless				Peeled and deveined			
	Temperature of incubation °C											
	5	5/30	RT	37	5	5/30	RT	37	5	5/30	RT	37
Initial	10.30	235.00	245.00	240.00	9.40	160.00	190.00	188.00	2.6	200.00	215.00	205.00
0	2.70	34.00	40.00	39.00	0.84	26.30	39.00	30.00	9.2	64.00	103.00	100.00
1	0.96	4.60	6.50	6.10	0.57	6.00	6.90	7.10	2.0	49.00	82.00	80.00
2	0.80	1.02	1.35	1.18	1.60	3.80	3.90	4.10	3.2	59.00	60.00	63.00
6	0.46	0.63	0.65	0.61	0.87	0.97	1.01	1.07	8.8	11.00	11.00	19.00
10	0.30	0.31	0.38	0.39	0.48	0.49	0.51	0.58	7.7	7.60	7.80	7.70
12	0.20	0.27	0.28	0.28	0.32	0.36	0.37	0.37	7.4	7.90	7.80	7.70

Note: 3 series of experiments were undertaken during the period August, 1983 to April, 1985. Comparable results were obtained typical results are presented here.

and PD at the end of the storage period were $2.8x10^2$/g - $3.4x10^2$/g, $3.7x10^2$/g - $4.7x10^2$/g and $2.2x10^2$/g - $8.2x10^2$/g respectively.

Typical results from the study are presented in Table 52 and 53. An identical bacterial count in SWA and TGA was noted in the case of mackerel during frozen storage. But, while the bacterial count was almost equal in two media till the end of frozen storage of mackerel, in the case of prawn, TGA was showing better count in many instances. This may be due to difference in the terminal flora of the two.

When the plates were incubated at the three temperatures viz. 5°C, RT (29±2°C) and 37°C, incubation at RT generally yielded higher bacterial count. This was true for prawn frozen as whole, headless or PD. Here also, bacterial flora differed from that of mackerel wherein throughout the period of frozen storage, a higher bacterial count was noticed at RT. In prawn, a higher count at 37°C was noted particularly towards the end.

The effect of incubation temperature of the plates on the TPC of prawn was similar to that observed for mackerel (Tables 54 and 55).

The percentage of psychrotrophs in prawn is presented in Table 56. The peeled and deveined sample had a lower

Table 54

The ratio of the bacterial count at 5°C to that of 5/30 for muscle of prawn M. dobsoni during freezing at -40°C as whole, headless and PD and storage at -20±2°C

Period of frozen storage (months)	Bacterial count on					
	Whole prawn		Headless prawn		P D prawn	
	SWA	TGA	SWA	TGA	SWA	TGA
Initial	0.07	0.04	0.05	0.06	0.02	0.01
0	0.18	0.07	0.04	0.03	0.10	0.14
1	0.21	0.20	0.42	0.09	0.42	0.40
2	0.30	0.78	0.84	0.42	0.74	0.54
4	0.58	0.77	0.92	0.88	0.80	0.71
6	0.90	0.73	0.92	0.89	0.90	0.88
10	0.83	0.95	0.98	0.98	0.89	0.98
12	-	-	0.94	0.86	0.88	0.94

Table 55

The ratio of the bacterial count at RT to that at 37°C for muscle of prawn M. dobsoni during freezing at -40°C as whole, headless and PD and storage at -20±2°C

Period of frozen storage (months)	Whole prawn		Headless prawn		PD prawn	
	Bacterial SWA	Count on TGA	Bacterial SWA	Count on TGA	Bacterial SWA	Count on TGA
Initial	1.00	1.02	1.01	1.15	1.00	1.04
0	1.46	1.05	1.52	1.30	1.23	1.03
1	1.30	1.06	1.09	0.97	1.03	1.02
2	1.01	1.14	1.02	0.95	1.02	0.95
4	0.96	1.01	1.01	1.00	0.98	1.01
6	0.97	0.97	1.00	0.94	0.91	0.57
10	0.92	0.97	1.02	0.96	1.02	1.01
12	1.00	0.75	0.97	1.01	1.05	0.98

Table 56

Psychrotrophic count at 5°C as percentage of count at RT for muscle of prawn M. dobsoni during freezing at -40°C as whole, headless and PD and Stored at -20±2°C

Period of frozen storage (months)	Whole prawn		Headless prawn		PD prawn	
	Bacterial Count(%) SWA	TGA	Bacterial Count(%) SWA	TGA	Bacterial Count(%) SWA	TGA
Initial	4.48	4.20	5.06	4.95	1.87	1.21
0	12.50	6.75	2.96	2.15	7.56	8.90
1	21.05	14.76	42.60	8.26	27.16	24.39
2	41.46	59.26	77.50	41.20	31.03	53.30
4	58.90	76.13	80.00	67.60	74.52	71.29
6	89.95	70.76	92.80	86.14	80.00	80.00
10	83.30	78.94	97.90	93.56	93.60	99.60
12	90.00	95.20	80.50	97.20	82.00	93.60

psychrotrophic count in the beginning. The psychrotrophic count in SWA was 4.48, 5.06 and 1.87 for whole, headless and PD prawn respectively. In TGA the values were 4.2, 4.95 and 1.21 respectively. The percentage of psychrotrophs increased with frozen storage and composed more than 90% of the total population towards the end. It was interesting to note that TGA recovered equal or greater percentage of psychrotrophs than SWA at the end of storage period. This pointed to the possibility of mesophiles adapted to low temperature constituting the psychrotrophic flora. This was also supported by the finding that the ratio of the counts at 5°C to that of 5/30 gradually increased to reach a value near unity. Since the growth of psychrotrophic bacteria was quite unlikely at -20±2°C, the rise should be due to the adaption of mesophiles to low temperature. The lowest minimum temperature reported for growth of bacteria is -5° to -7°C and below -10°C to -12°C growth occurs very rarely (Ingram and Mackey, 1976).

The immediate effect of freezing on the bacteria present in prawn was a decline in their total population. The reductions due to freezing were 83.98% for prawn frozen as whole, 79.48% for headless and 53.1% for PD. Thus, there was lesser reduction in the total bacterial numbers in PD than the other two.

The decrease in number of bacteria during frozen storage was less especially after the first month. While 83.98% of bacteria was destroyed in prawn frozen as whole by the freezing process, the percentage of bacteria surviving after one year was 0.08%. In prawn frozen as headless, the percentage of survivors was 0.19% and in PD, 3.6%. This also shows that PD prawn exhibited a lower reduction in bacterial count during freezing. There was lesser reduction during storage also. The total reduction in bacterial count that occurred at the end of one year in the whole and headless prawns were almost comparable (Table 57).

The decrease in the bacterial number occurring during frozen storage between two storage periods at fixed intervals are presented in Table 58. In frozen prawn, frozen as whole, the rate of decrease during frozen storage up to 2 months was almost equal to that after freezing. After 2 months the decrease was less, irregular and ranged from 26.14% to 44.4% in whole,

Table 57

Survival of bacteria on the muscle of prawn, M. dobsoni during freezing at -40°C as whole, headless and PD and storage at -20±2°C

Period of frozen storage (months)	Percentage surviving in		
	Whole prawn	Headless prawn	PD prawn
0	17.02	20.52	46.99
1	2.76	3.63	38.13
2	0.57	2.05	27.90
4	0.37	1.37	16.27
6	0.26	0.53	6.51
10	0.16	0.27	3.62
12	0.08	0.19	3.62

Table 58

Reduction in the bacterial count of prawn during freezing and frozen storage

Period of frozen storage (months)	Percentage of reduction on		
	Whole prawn	Headless prawn	P and D prawn
Reduction after freezing	83.98	79.48	53.10
Reduction in first month	84.75	82.31	21.29
Reduction between 1 & 2 months	79.24	43.48	26.83
Reduction between 2 & 4 months	34.81	33.34	41.60
Reduction between 4 & 6 months	26.14	61.15	69.37
Reduction between 6 & 10 months	39.69	49.51	29.10
Reduction between 10 & 12 months	44.44	27.46	-

Note: Prawn M. dobsoni frozen at -40°C as whole, headless and P & D and stored at -20±2°C for 1 year. The percentage of reduction due to freezing is the reduction from initial count of prawn muscles before freezing. Percentage of reduction after storage is from bacterial count of the prawn muscle from just previous period.

27.4% -61.15% in headless and 21.39% - 49.57% in PD. According to Ingram and Mackey (1976), the changes developing during frozen storage in solute concentration, pH or ice pattern may result in relatively irregular decline of bacterial numbers with time.

The total plate count reported for frozen headless prawn, collected from processing factories at Cochin, ranged from $1.0x10^4$/g - $1.0.10^5$/g at 37°C. For PD prawn, the counts were in the range of $1.0x10^4$/g to $1x10^7$/g (Pillai et al., 1965). For factory processed shrimp from Thailand, the bacterial count ranged from $1.0x10^6$/g - $8.3x10^7$/g at 20°C and from $3.0x10^5$/g - $3.9x10^7$/g at 37°C (Cann, 1974). Bacteriological examination of factory processed Malayasian shrimp showed total plate count of $1.0x10^6$/g for PD and $4.0x10^6$/g for cooked and peeled (Liston, 1980). Shrimp from temperate water showed a bacterial count of $5.3x10^3$/g - $2.7x10^3$/g at 37°C and for Scampi, a bacterial count of

$3.8x10^5$/g to $2.8x10^7$/g prevailed (Hobbs et al. 1971). The total plate counts reported in this study, is found to be comparable to the above figures.

A higher bacterial count at ambient temperature was noted in several instances. A similar finding has been reported for tropical shrimp (Cann, 1974; Kawabata et al., 1975) as well as temperate types (Hobbs et al., 1971).

According to the present study a higher bacterial count after freezing was noticed in PD prawn which was followed by headless and whole prawns. A similar finding was reported by Pillai et al. (1965) and they attributed the reason to larger meat surface area of the prawn exposed to bacterial attack. But it is also possible that a change in the bacterial flora pattern occurring due to greater handling can also lead to such results.

Thus, the overall reduction due to freezing was 72 to 82% for prawn frozen as whole, 76 to 82% for headless prawn and 32 to 53% for peeled and deveined prawn. The reduction in bacterial number by freezing reported previously (Shewan, 1961; Lakshmy et al., 1962; Novak, 1973; Liston, 1980) agrees with this finding. Whereas Novak (1973) reported uniform reduction during freezing

and at definite intervals of frozen storage, in the present investigation such behaviour was noticed only in the early period of frozen storage. Similar pattern was noted in the case of freezing and frozen storage of mackerel also. In pure culture studies in early period of freezing and frozen storage, where high cell concentration prevailed, there was the existence of population effect and this disappeared at low cell concentrations. The same may be applicable here also.

The incidence of pathogenic/indicator bacteria in prawn was screened throughout the period of frozen storage. (Table 59). The values showed that the incidence of pathogens/indicator microorganisms were very low in the sample of prawn. The values presented here were comparable to that of Cann (1974) and Liston (1980).

The organoleptic scoring were done side by side with the bacteriological and chemical analysis and are presented in Table 60 and Figures 33.1 to 33.4 respectiyely. While bacterial count went on decreasing with freezing and frozen storage, samples remained organoleptically in good condition for 6-8 months in the three types of prawn. There was also no correlation of bacterial number with chemical indices or organoleptic qualities during storage at -20±2°C. . similar observations have been noted previously by Pillai et al.(1965) and Liston, (1980).

Table 59

Quantitative changes in the pathogenic/indicator bacteria in the muscle of prawn, M. dobsoni during freezing at -40°C and storage at -20±2°C

Period of storage (months)	Type	Salmonella	Coliforms	E. coli	Staphylococci	Streptococci	Vibrio pharahemolyticus
Initial	W	Nil	1092*	637	237	$2.1x10^3$	Nil
	HL	"	$1.4x10^3$	858	391	$2.3x10^3$	"
	PD	"	716	55	134	$2.1x10^3$	"
0	W	"	115	Nil	172	$9.3x10^2$	"
	HL	"	211	93	184	$8.2x10^2$	"
	PD	"	216	72	91	$1.2x10^3$	"
1	W	"	Nil	Nil	Nil	473	"
	HL	"	"	"	94	472	"
	PD	"	"	"	Nil	650	"
2	W	"	"	"	"	267	"
	HL	"	"	"	"	288	"
	PD	"	"	"	"	188	"
6	W	"	"	"	"	Nil	"
	HL	"	"	"	"	"	"
	PD	"	"	"	"	"	"
10	W	"	"	"	"	"	"
	HL	"	"	"	"	"	"
	PD	"	"	"	"	"	"
12	W	"	"	"	"	"	"
	HL	"	"	"	"	"	"
	PD	"	"	"	"	"	"

*Count/g of the muscle; W - stands for whole prawn; H - for headless and PD for peeled and deveined.

Table 60

Changes in the organoleptic characteristics of prawn M. dobsoni after freezing at -40°C and frozen storage at -20±2°C for one year

Period of frozen storage (months)	Whole prawn	Score	Headless prawn	Score	Peeled and deveined prawn	Score
0	Excellent	9	Excellent	9	Excellent	9
1	Very good	8	Very good	8	Very good	9
2	Good	7	Good	7	Good	7
4	Good	7	Good	7	Good	7
6	Good slightly tough	5	Good slightly tough	5	Good	6
10	Poor tough	4	Poor	4	Poor	4
12	Poor, very tough	3	Poor, very tough	3	Poor, very tough	3

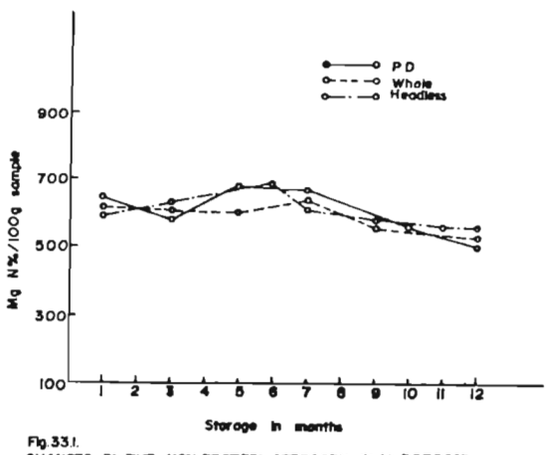

Fig.33.1.
CHANGES IN THE NON-PROTEIN NITROGEN IN M. DOBSONI AFTER FREEZING AT -40°C AND STORAGE AT -20±2°C

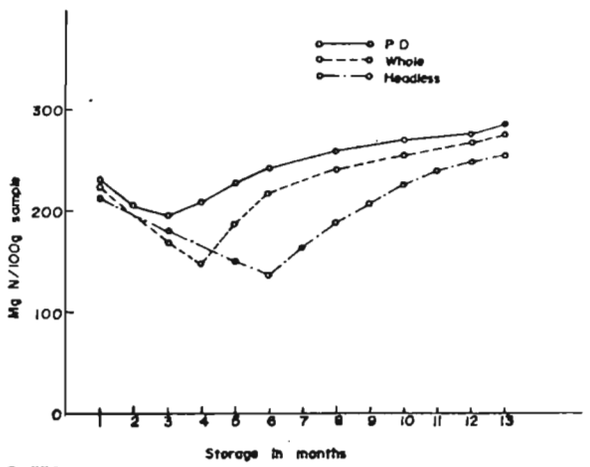

Fig.33.2.
CHANGES IN α-AMINO NITROGEN IN M. DOBSONI AFTER FREEZING AT -40°C AND STORAGE AT -20±2°C

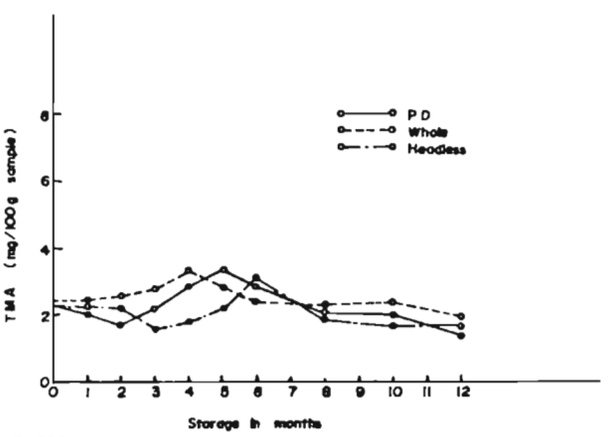

Fig.33.3.
CHANGES IN TRIMETHYLAMINE IN M. DOBSONI AFTER FREEZING AT -40°C AND STORAGE AT -20±2°C

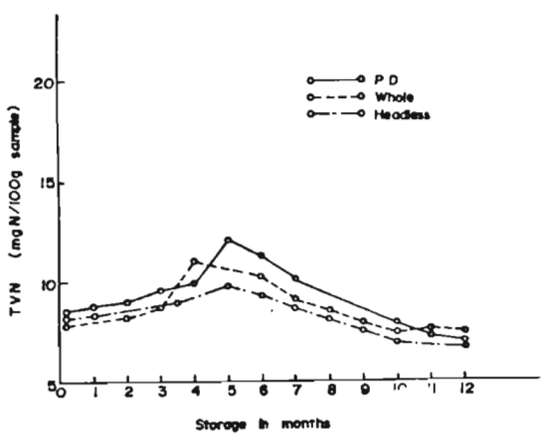

Fig.33.4.
CHANGES IN TOTAL VOLATILE NITROGEN IN M. DOBSONI AFTER FREEZING AT -40°C AND FROZEN STORAGE AT -20±2°C

4.4.2.2.2 Qualitative changes

The changes in the distribution of major bacterial groups in the muscle of prawn M. dobsoni, frozen as whole, headless and PD at -40°C and stored at -20±2°C are presented (Tables 61, 62 and 64). Only TGA had been used as the recovery media for frozen prawn. The effect of incubation temperature of plates on the bacterial flora was also studied.

The most salient change of freezing and frozen storage was a gradual shift in the nature of bacterial population from the Gram-negative types to Gram-positives. For prawn, processed as whole, headless and PD the percentage of Gram-positives at RT before freezing were respectively 23, 29 and 53. After freezing the values rose to 42, 37 and 60. After 6 months of frozen storage at -20±2°C 63% of bacteria in the prawn, frozen as whole, 60% of them in headless and 64% in PD were composed of Gram-positives. At the end of frozen storage, nearly 75% of the total bacterial population was composed of Gram-positives.

The Gram-positive flora was mainly constituted by the family Micrococcaceae in which the major genera were Micrococcus, Staphylococcus and Planococcus. No attempt was made to identify them up to species level. The

Table 61

Qualitative changes in the major microbial groups in the muscle of prawn (whole) during freezing at -40°C and frozen storage at -20±2°C

Period of frozen storage (months)	Temperature of incubation °C	Pseudomonas	Acinetobacter	Moraxella	Vibrio	Flavobacterium/cytophaga	Micrococcus	Bacillus	Arthrobacter	Others	Total isolates
Initial	5	20*	16	17	12	8	19	2	4	2	84
	RT	20	16	17	14	6	20	1	2	4	75
	37	20	17	16	14	3	25	2	2	1	85
0	5	12	16	15	3	6	46	1	1	0	97
	RT	14	18	20	5	1	37	1	4	0	77
	37	6	20	22	6	1	40	2	3	0	79
1	5	14	18	22	0	0	45	1	0	0	87
	RT	13	13	19	0	0	52	1	1	1	84
	37	5	18	12	0	1	53	1	1	1	86
2	5	13	20	19	0	0	48	0	0	0	84
	RT	12	15	20	0	0	53	0	0	0	73
	37	5	20	18	0	2	54	1	0	0	89
6	5	12	13	15	0	0	60	0	0	0	89
	RT	9	10	18	0	0	63	0	0	0	92
	37	2	13	20	0	0	65	0	0	0	82
10	5	8	12	15	0	0	65	0	0	0	90
	RT	4	14	15	0	0	67	0	0	0	91
	37	2	13	16	0	0	68	0	0	1	77
12	5	2	11	21	0	0	66	0	0	0	78
	RT	0	15	16	0	0	69	0	0	0	93
	37	1	14	13	0	0	72	0	0	0	80

*Expressed as percentage of total isolates

Table 62

Qualitative changes in the major microbial groups in the muscle of prawn (headless) during freezing and frozen storage

Period of frozen storage (months)	Temperature of incubation °C	Pseudomonas	Acinetobacter	Moraxella	Vibrio	Flavobacterium/cytophaga	Micrococcus	Bacillus	Arthrobacter	Others	Total isolates
Initial	5	23*	16	18	15	4	19	2	2	1	92
	RT	20	17	14	15	3	24	2	3	2	75
	37	24	15	17	15	4	23	1	1	0	92
0	5	12	23	24	6	2	23	2	3	5	90
	RT	15	21	22	5	1	35	1	1	1	74
	37	7	13	22	5	2	48	2	1	0	91
1	5	13	19	21	0	0	47	0	0	0	89
	RT	13	17	21	0	0	50	0	1	0	81
	37	5	12	18	0	2	55	2	2	4	94
2	5	14	18	20	0	0	48	0	0	0	71
	RT	10	15	24	0	0	51	0	0	0	77
	37	6	15	20	0	0	61	1	1	0	87
6	5	10	14	16	0	0	60	0	0	0	94
	RT	6	16	17	0	0	60	1	0	0	94
	37	2	14	19	0	0	64	1	0	0	90
10	5	8	10	17	0	0	65	0	0	0	86
	RT	0	15	17	0	0	68	0	0	0	88
	37	0	13	19	0	0	68	0	0	0	75
12	5	2	11	18	0	0	69	0	0	0	82
	RT	0	15	14	0	0	71	0	0	0	92
	37	0	16	14	0	0	70	0	0	0	81

*Expressed as percentage of total isolates

Table 63

Qualitative changes in the major microbial groups in the muscle of prawn (peeled and deveined) during freezing and frozen storage

Period of frozen storage (months)	Temperature of incubation °C	Pseudomonas	Acinetobacter	Moraxella	Vibrio	Flavobacterium/ cytophaga	Micrococcus	Bacillus	Arthrobacter	Others	Total isolates
Initial	5	6*	12	14	13	5	47	1	2	0	92
	RT	8	11	10	10	4	46	3	4	4	72
	37	5	11	11	13	4	50	2	2	2	72
0	5	4	20	21	0	0	51	0	2	2	88
	RT	2	17	14	5	2	50	2	8	0	70
	37	6	14	16	3	2	54	1	2	2	81
1	5	3	24	17	0	0	54	0	0	2	89
	RT	8	20	16	0	2	50	0	2	2	84
	37	4	15	28	0	1	50	2	2	0	89
2	5	2	20	18	0	0	56	0	0	4	91
	RT	2	22	20	0	0	52	1	3	0	79
	37	2	18	24	0	1	55	0	0	0	83
6	5	4	18	27	0	0	50	0	1	0	75
	RT	2	13	15	0	0	64	0	0	0	63
	37	3	13	20	0	0	64	0	0	0	84
10	5	2	13	17	0	0	68	0	0	0	80
	RT	3	10	12	0	0	71	0	2	2	86
	37	0	10	15	0	0	73	1	0	1	90
12	5	1	10	18	0	0	71	0	0	0	94
	RT	0	12	8	0	0	76	0	0	4	73
	37	0	9	14	0	0	75	0	0	2	78

*Expressed as percentage of total isolates

percentage of this Gram-positive group was greater in PD prawn after freezing and subsequent storage at -20±2°C. The other Gram-positives viz. Bacillus and Arthrobacter were fewer in number compared to the Micrococcus group and ranged from 1-2% and 2-4% respectively at RT.

The Gram-negative flora contained in raw prawn just before freezing were mainly composed of Pseudomonas, Moraxella, Acinetobacter, Vibrio and Flavobacterium/ cytophaga species. The bacterial flora of whole prawn was almost comparable to that of headless, while PD prawn harboured totally different flora composition. Thus, the percentage of the Pseudomonas in whole, headless and PD prawn at RT, were 20, 20 and 8, Moraxella 17, 14 and 10, Acinetobacter 16, 17 & 11 and Vibrio 14, 15 and 10 respectively. However, after freezing there was a change in all these types. The percentage of Pseudomonas in whole, headless and PD came down to 14, 15 and 6. Moraxella rose to 20, 22 and 14, so also Acinetobacter, to 18, 21 and 17 and Vibrio decreased to 5, 6 and 5 respectively. Frozen storage for one month resulted in further fall in the number of Pseudomonas and Vibrio, while Moraxella and Acinetobacter remained more or less same. There was a total elimination of Vibrio from the system after one month storage. This showed that effect

of freezing and frozen storage among the Gram-negative bacteria differed. Thus the Moraxella spp. was the most resistant, Acinetobacter less so and Vibrio most sensitive.

The effect of incubation of the plates on the selection of the flora of the prawn was also evident. Effect in this case was similar to that of mackerel.

Eventhough voluminous reports have come on the bacteriological studies of frozen prawn (Raj, 1970; Pillai et al., 1965; Hobbs et al., 1971; Cann, 1974; Gjerde, 1976; Baer et al., 1976; Karunasagar et al., 1984), their data has stressed the changes in the total bacterial counts and also counts of pathogenic/indicator bacteria. Information on the changes in the individual species comprising the total population or their mortality during freezing process is very limited.

The bacterial flora for imported frozen tropical shrimp, reported by Kawabata et al. (1975), bear similarities with the present results. They reported 70% of the flora of frozen shrimp to be composed of Gram-positives belonging to the genera Micrococcaceae. The Gram-negative fraction was constituted by Pseudomonas (14%), Moraxella (45-47%) and Flavobacterium/Cytophaga. The recovery of Moraxella at 37°C was 16-27% and Micrococcaceae 56-70% as against 45-47% and 64% at 25°C respectively.

The results of this study reveal that the data regarding the survival during freezing of marine pathogenic and non pathogenic bacteria in fish muscle medium is in good agreement with the data on their survival in whole fish/prawn.

The Pseudomonas, Salmonella and E. coli strains which were very sensitive to freezing temperatures suffered rapid destruction in whole fish/prawn. On the otherhand, the Micrococcus spp. which were very resistant to freezing temperatures continued to survive till the end of frozen storage. Vibrio spp., the most sensitive of all bacterial strains to freezing temperatures, were eliminated from whole fish in the early period of frozen storage.

However, the Moraxella spp., though as sensitive as Pseudomonas to freezing temperatures were retained in whole prawn for longer periods. Similar behaviour was observed in the case of Acinetobacter spp. as well.

Since Moraxella could survive better in fish muscle medium at freezing temperatures along with Pseudomonas spp., (4.3.2.1), it may be argued that the nature of the component flora may be a determinating for survival at low temperature. As pointed out by Karunasagar et al. (1985), the environment existing in fish muscle medium may

be different from that of whole prawn/fish due to the absence of compounds like chitin and may cause variations.

4.4.3 Statistical analysis of the data

Since the total bacterial count after each successive step varied along with variation in the qualitative nature of the flora a regression analysis of the two was carried out.

The data were analysed using the method of simple regression analysis (Snedecor and Cochran, 1961) by taking the months of storage as X and logarithm of bacterial count as y. In the case of Psuedomonas, Moraxella, Acinetobacter and Micrococcus, the percentage of each to the total count were taken as Y. The significance of the regression coefficient (b) of months of storage on bacterial count were tested using 't' test;

$$t = \frac{b}{Sb}$$

where b is the regression coefficient and Sb is the standard error of b. The degrees of freedom of 't' is (n-2) where 'n' is the number of months of storage.

The results of the regression analysis are shown in Tables 64 to 67. Table 64 gives the regression analysis of the changes in the bacterial load in different stages of preprocessing of mackerel and Table 65, that of prawn.

Table 64

Regression analysis of bacterial load in Mackerel - preprocessing stages

	xy	x^2	y^2	b	n	Sy.X	Sb	1t1	df	P
Log total count	-1.4826	5	0.6658	-0.2965	4	0.3363	0.1504	1.970	2	NS
Pseudomonas	-8.0000	5	29.0000	-1.6000	4	2.8460	1.2728	1.257	2	NS
Moraxella	-18.0000	5	66.0000	-3.6000	4	0.7746	0.3464	10.393	2	0.01
Vibrio	-32.0000	5	228.0000	-6.4000	4	3.4059	1.5231	4.202	2	NS
Micrococcus	32.5000	5	222.7500	6.5000	4	2.3979	1.0724	6.061	2	0.05
Flavobacterium	7.0000	5	22.0000	-0.6000	4	2.4698	1.1045	0.543	2	NS

b = regression coeft., n = sample size; Sb = standard error of b; df = degrees of freedom; p = level of significance; NS = not significant at 5% level

Table 65

Regression analysis of bacterial load in *M. dobsoni* - preprocessing stages

	xy	x^2	y^2	n	b	Sy.x	Sb	ltl	df	P
Log total count	-1.9582	17.5	1.2119	6	-0.1119	0.4982	0.1191	0.940	4	NS
Pseudomonas	-51.0000	17.5	268.0000	6	-2.9143	5.4629	1.3059	2.232	4	NS
Moraxella	-31.5000	17.5	70.8333	6	-1.8000	1.8797	0.4493	4.006	4	0.01
Acineto-bacter	-7.0000	17.5	15.3333	6	-0.4000	1.7701	0.4231	0.945	4	NS
Vibrio	-39.5000	17.5	347.5000	6	-2.2571	8.0365	1.9211	1.175	4	NS
Micrococcus	124.0000	17.5	1107.3333	6	+7.0857	7.5615	1.8075	3.920	4	0.01
Flavo-bacterium	-15.0000	17.5	31.3333	6	-0.8571	2.1492	0.5138	1.668	4	NS

b = regression coeft., n = sample size; Sb = standard error of b; df = degrees of freedom; p = level of significance; NS = not significant at 5% level

Table 66

Regression analysis of bacterial count in mackerel during freezing and frozen storage

	xy	x^2	y^2	n	b	sy·x	Sb	ltl	df	p
Log TPC	-22.354327	147.726503	3.878371	8	-0.1513	0.287420	0.0236480	6.398	6	$p < 0.001$
Pseudomonas	-219.063375	147.726503	545.875000	8	-1.4829	6.069408	0.4993630	2.970	6	$p < 0.05$
Moraxella	89.571875	147.726503	134.875000	8	0.6063	3.664340	0.3014060	2.011	6	NS
Acinetobacter	130.292125	147.726503	288.875000	8	0.8820	5.384541	0.4410160	1.990	6	NS
Micrococcus	247.143750	147.726503	467.500000	8	1.6730	3.000917	0.2469030	6.776	6	$p < 0.001$
Flavobacterium	-60.491500	147.726503	32.000000	8	-0.4095	1.097706	0.0903140	4.534	6	$p < 0.01$

b = regression coeft., n = sample size; Sb = standard error of b; df = degrees of freedom;
p = level of significance; NS = not significant at 5% level

Table 67

Regression analysis of bacterial count in prawn *M. dobsoni* during freezing and frozen storage

		xy	x^2	y^2	n	b	sy.x	Sb	|t|	df	p
Log bacterial count	W	-32.2485	159.3934	9.3934	9	-0.2023	0.9654	0.0765	2.632	7	<0.05
	HL	-28.2948	159.3934	6.4313	9	-0.1775	0.4486	0.0355	4.996	7	<0.01
	PD	-33.7446	159.3934	10.2646	9	-0.2117	0.6677	0.0529	4.003	7	<0.01
Pseudomonas	W	-126.5423	159.3934	133.5556	9	-0.7939	2.1743	0.1722	4.610	7	<0.01
	HL	-255.7283	159.3934	466.2222	9	-1.6044	2.8268	0.2239	7.166	7	<0.001
	PD	-77.1013	159.3934	82.8889	9	-0.4837	2.5521	0.2021	2.393	7	<0.05
Moraxella	W	30.4573	159.3934	103.5566	9	0.2941	3.7366	0.2960	0.994	7	NS
	HL	-67.8473	159.3934	174.2222	9	0.4257	4.5567	0.3609	1.179	7	NS
	PD	-68.6103	159.3934	175.5556	9	-0.4304	4.5985	0.3642	1.182	7	NS
Acinetobacter	W	-70.7967	159.3934	68.8889	9	-0.4442	2.3128	0.1832	2.425	7	<0.05
	HL	-14.9150	159.3934	46.0000	9	-0.0936	2.5243	0.1999	0.468	7	NS
	PD	-110.2110	159.3934	206.0000	9	-0.6918	4.3047	0.3410	2.029	7	NS
Micrococcus	W	362.5420	159.3934	966.0000	9	2.2745	4.4943	0.3560	6.389	7	<0.001
	HL	491.9143	159.3934	1900.8889	9	3.0862	7.3946	0.5857	5.269	7	<0.01
	PD	421.6437	159.3934	1202.8889	9	2.6453	3.5358	0.2801	9.444	7	<0.001

b = regression coefficient; n = sample size; Sb = standard error of b; df = degree of freedom, p = level of significance; NS not significant at 5% level

This showed that for both mackerel and prawn, Moraxella showed significant rate of decrease ($p < 0.01$) and Micrococcus showed significant rate of increase ($p < 0.01$). Total plate count and counts of Pseudomonas, Vibrio and Acinetobacter did not show significant rate of decrease at 5% level.

The regression analysis of mackerel during freezing and frozen storage is given in Table 66. It was observed that logarithm of the total count and percentage of Pseudomonas showed significant rate of decrease ($p < 0.01$ and $p < 0.05$ respectively). The Micrococcus showed a significant rate of increase ($p < 0.1$) with storage period. Moraxella and Acinetobacter species did not show any significant rate of decrease at 5% level.

Table 67 shows the regression analysis of prawn during freezing and frozen storage. Pseudomonas showed a significant decrease in prawn frozen as whole ($p < 0.05$), headless ($p < 0.001$) and PD ($p < 0.01$). Moraxella did not show any significant change at 5% level. But the Micrococcus species showed a significant increase in whole ($p < 0.01$), headless ($p < 0.01$) and PD ($p < 0.001$) prawn. Acinetobacter showed a significant decrease ($p < 0.05$) in PD, while in the other two types there was no significant change at 5% level.

4.4.4 Changes in the physiological groups of bacteria in fish R. kanagurta and prawn M. dobsoni during freezing and frozen storage

4.4.4.1 Changes in the physiological groups in mackerel R. kanagurta

Table 68 represents the changes in the distribution of various physiological groups of bacteria in the skin and muscle of mackerel (Rastrelliger kanagurta) during freezing at -40°C and storage at -20±2°C for up to one year. The effect of temperature on the biochemical activity is also studied.

Gelatin liquefaction was used to test for proteolytic activity. The changes in the gelatin liquefiers showed a downward trend with freezing and frozen storage i.e. from 52% before freezing to 2% after one year. Bacteria capable of producing indole were very low in number in mackerel before freezing and practically nil after freezing. The production of hydrogen sulphide (H_2S) was observed in fairly good number of isolates and their number, reaching peak value after 4 months, came down to initial levels after storage for one year. The percentage of nitrate-reducing bacteria was also prominant and remained so during freezing and storage. Bacteria capable of hydrolysing starch were few in number and their number decreased with storage. Contrary to it, the pigmented bacteria showed

Table 68

Changes in the various physiological groups of bacteria in mackerel during freezing and storage

Type	Incubation temperature °C	Percentage of the total isolates							
		Period of storage in months							
		Initial	0	1	2	4	6	10	11
Gelatin liquefiers	5	50	40	9	8	9	2	2	3
	RT	52	40	12	10	9	2	1	2
	37	50	40	10	10	9	0	1	0
Indole producers	5	3	0	0	0	0	0	0	0
	RT	12	2	0	0	0	0	0	0
	37	14	2	0	0	0	0	0	0
H_2S producers	5	45	64	40	60	52	40	42	35
	RT	45	64	45	70	72	40	44	36
	37	45	64	46	68	72	41	44	36
Nitrate reducers	5	48	56	50	56	68	62	65	63
	RT	48	60	50	58	67	80	67	62
	37	50	60	48	58	67	78	70	62
Amylase producers	5	6	8	8	7	6	5	0	0
	RT	16	16	10	5	3	2	0	0
	37	18	16	15	6	3	2	0	0
Pigmented bacteria	5	1	10	9	8	8	4	6	7
	RT	7	50	55	49	48	48	43	39
	37	8	50	55	50	48	50	44	40
Utilization of glucose	5	16	50	52	50	48	40	45	48
	RT	16	52	60	54	52	42	46	50
	37	15	50	54	54	50	40	43	47

Note: Fifty cultures were isolated in each case and subjected to tests

significant increase at RT from 7% before freezing to 50% after freezing and remained more or less stationary. The percentage of bacteria, which were capable of producing acid from glucose, also followed identical pattern.

4.4.4.2 Changes in the physiological groups of bacteria in prawn M. dobsoni

The changes in the physiological groups in muscle of prawn M. dobsoni during freezing at -40°C and storage at -20±2°C are presented in Table 69.

The gelatin liquefaction was observed in 34% of the isolates at RT before freezing and their number came to 2% after one year storage at -20±2°C. Thus, it showed a downward trend with freezing and frozen storage. Production of hydrogen sulphide, production of acid from glucose and hydrolysis of starch (amylase production) followed similar trends. The percentage of cultures producing H_2S, acid and amylase at RT were respectively 20, 43 and 20 in the material. After storage for 6 months, the corresponding values were 8, 10 and 2. Indole producing types, though present initially, were eliminated after storage for 2 months, but the number of bacteria reducing nitrate remained stationary. The number of pigmented bacteria showed rapid increase, from 6% before freezing to 70% after 6 months.

Table 69

Changes in the physiological groups of bacteria in prawn during freezing and storage

Type	Incubation temperature °C	Percentage of the total isolates							
		Period of storage in months							
		Initial	0	1	2	4	6	10	12
Gelatin liquefiers	5	36	26	19	15	7	0	2	0
	RT	34	28	19	15	8	4	2	2
	37	34	28	20	15	7	5	1	1
Indole producers	5	25	6	0	0	0	0	0	0
	RT	30	6	1	0	0	0	0	0
	37	42	10	2	0	0	0	0	0
H_2S producers	5	19	28	14	12	6	8	6	7
	RT	20	28	20	15	7	8	7	7
	37	20	28	21	16	7	7	8	8
Nitrate reducers	5	50	48	60	61	60	62	60	55
	RT	56	62	68	63	64	62	65	60
	37	59	62	68	63	65	62	66	61
Amylase producers	5	12	10	8	6	6	5	0	0
	RT	20	18	12	8	6	2	0	0
	37	24	20	12	8	6	0	0	0
Pigmented bacteria	5	2	2	4	10	11	8	10	12
	RT	6	63	64	66	70	70	69	68
	37	11	68	67	66	73	71	70	72
Bacteria utilizing glucose	5	42	23	16	15	16	12	9	8
	RT	43	25	18	12	12	10	10	9
	37	45	24	18	14	9	9	8	8

Note: Fifty cultures were isolated in each case and subjected to the tests

Eventhough fresh mackerel carried a greater percentage of gelatin liquefying bacteria (52%) than the prawn (34%), after freezing and frozen storage, their numbers became almost equal. Similarly, bacteria capable of producing indole was greater in prawn, but on storage this type disappeared totally in both fish and prawn. The number of bacteria capable of producing H_2S were of lower order in prawn compared to mackerel. While this type showed a downward trend in prawn, in mackerel they remained stationary. A similar pattern was observed in the case of production of acid from glucose. The amylase production and reduction of nitrate was found in almost similar pattern in both mackerel and prawn. The percentages of pigmented cultures were greater in prawn. While the number of pigmented bacteria in mackerel decreased slightly on storage from 50% to 39%, that of prawn increased from 63% to 68%.

There was wide variation in the prevalance of various physiological groups of bacteria in fish and prawn during freezing and subsequent frozen storage. Contrasting results were observed in many instances as in the case of production of acid from glucose and production of H_2S. The selective action of freezing might have caused an accumulation of one or few bacterial species during frozen storage resulting in the predominance

of certain biochemical groups of bacteria.

Out of the 52 cultures which liquefied gelatin at RT, all except two liquefied gelatin at 5°C. While these cultures liquefied gelatin in 3-4 days at RT, at 5°C it required 15-21 days. Similarly, the time for acid production was 24 hours at RT or 37°C and 5-6 days at 5°C. This showed that temperature of incubation had an effect not on biochemical activity, but on the rate of it. This is supported by the statement of Hess (1934) and Kiser (1944) that at low temperature, biochemical activities of the organisms are retained to some extent, but the reaction rate is strikingly altered. Kiser (1944) also noted that near the minimum temperature of growth of a particular organism , all the characteristic physiological properties of the microorganism, which occur at its optimum temperature of growth, would not operate.

The percentage of pigmented bacteria recovered at any period during freezing and frozen storage was greater at 37°C or RT than 5°C. This was true for both prawn and fish. A similar observation had been made by Ayres (1960) during refrigerated storage of beef. He observed that the ability to produce the pigment is temperature - dependent and incubation at higher

temperature or aeration could restore the pigment in a culture which lost the pigmentation during low temperature incubation. This may be reason for the occurrence of lesser proportion of pigmented bacteria at 5°C compared to RT or 37°C.

It may be concluded that distinct changes occur in physiological groups of bacteria due to freezing and frozen storage of mackerel and prawn. According to Rey et al. (1969), most of the metabolically active organisms in poultry survived freezing to a greater extent than the less active ones. But according to the data presented here, no relationship could be derived between biochemical activity and resistance to frozen storage. While some of the biochemically active groups increased with frozen storage, others decreased in proportion. This study also shows that the nature of substrate is also very important, since different results were obtained in the two substrates fish and prawn, during freezing and frozen storage.

4.4.5 Effect of thawing on the bacterial flora of frozen mackerel *R. kanagurta* and prawn *M. dobsoni*

4.4.5.1 Quantitative aspects

4.4.5.1.1 Quantitative changes in the bacterial flora of mackerel R. kanagurta during thawing

The changes occurring in the total bacterial population of frozen mackerel, frozen at -40°C and stored at -20±2°C for four months were studied at three thawing temperatures viz. +4°C, +15°C and room temperature (29±2°C). The results are presented in Table 70. The effect of incubation temperature of the plates on the recovery of the bacteria during thawing was also investigated.

A control sample of fresh, uniced mackerel was kept side by side with the frozen material at the three thawing temperatures viz. +4°C, +15°C and RT.

As seen from the data, frozen mackerel when thawed at +4°C, the lowest of thawing temperature, produced least counts. Eventhough the zero hour count at RT was very identical for the three samples, the count after 24 hours of thawing at +4°C, +15°C and RT of frozen mackerel were respectively 6.18×10^3/g, 5.71×10^3/g and 5.28×10^7/g. The mean generation time at RT, of total bacterial population in the frozen mackerel thawed at RT, +15°C and +4°C, were 1.8 h, 3.46 h and 39.34 h respectively. This showed that lower temperature of thawing arrested the bacterial growth and multiplication in frozen fish.

Table 70

**Changes in the total bacterial count of frozen mackerel R. kanagurta (frozen at -40°C and stored for 4 months at -20±2°C) during thawing at different temperatures

Thawing time (hours	Thawing temperature °C	TPC of frozen mackerel			TPC of unfrozen mackerel		
		Temperature of incubation °C			Temperature of incubation °C		
		5	RT	37	5	RT	37
0	-	2.11×10^{3}*	5.18×10^{3}	2.37×10^{3}	1.2×10^{3}	2.11×10^{4}	7.8×10^{3}
2	4	2.00×10^{3}	5.60×10^{3}	2.17×10^{3}	1.6×10^{3}	1.00×10^{4}	1.04×10^{4}
	15	7.12×10^{3}	9.4×10^{3}	9.10×10^{4}	1.1×10^{4}	9.80×10^{3}	8.90×10^{3}
	RT	9.90×10^{4}	1.1×10^{4}	1.70×10^{4}	5.3×10^{4}	6.40×10^{4}	6.20×10^{4}
9	4	2.10×10^{3}	5.4×10^{3}	2.20×10^{3}	2.0×10^{3}	9.8×10^{3}	9.00×10^{3}
	15	1.08×10^{4}	1.2×10^{4}	1.20×10^{4}	3.2×10^{5}	1.2×10^{6}	1.10×10^{6}
	RT	5.80×10^{4}	7.2×10^{4}	7.70×10^{4}	6.8×10^{5}	1.8×10^{6}	2.10×10^{6}
16	4	1.90×10^{3}	4.8×10^{3}	2.30×10^{3}	4.0×10^{3}	8.6×10^{3}	9.20×10^{3}
	15	6.50×10^{4}	7.8×10^{4}	8.10×10^{4}	1.6×10^{5}	3.8×10^{5}	2.90×10^{5}
	RT	3.10×10^{6}	3.7×10^{6}	2.90×10^{6}	7.8×10^{5}	4.2×10^{8}	5.7×10^{8}
24	4	3.10×10^{3}	6.8×10^{3}	5.43×10^{3}	9.2×10^{3}	1.4×10^{4}	9.9×10^{4}
	15	3.60×10^{5}	5.71×10^{5}	5.68×10^{6}	9.12×10^{5}	1.1×10^{7}	9.8×10^{7}
	RT	1.9×10^{7}	5.21×10^{7}	5.28×10^{7}	9.20×10^{6}	1.1×10^{9}	9.3×10^{8}

*Average of triplicate plates

**Three series of experiments were undertaken and comparable results were obtained. Data presented here represents results of a typical series

In control samples of fresh uniced fish, the mean generation time of total bacterial population at +4°C, +15°C and RT were 1.22 h, 3.7 h and 25.5 h respectively, on the basis of bacterial count at RT. The frozen mackerel, with an initial bacterial count of 5.18×10^3/g at RT, when thawed at room temperature, reached a level of 5.21×10^7/g in 24 h. To reach approximately the same level in raw fish, the time required was between 9 and 16 h. This showed that there was a lag in growth of bacteria in frozen fish during thawing at room temperature and that the lag period was 8 to 16 h. Similar lag period observed for frozen mackerel during thawing at +15°C was about 8 h. At +4°C, the effect of lag period was longer and very distinct and was about 24 h at +4°C.

The drip which exuded from the fish, during thawing was studied for changes of total bacterial count (Table 72). The total bacterial count of the muscle and thaw drip during thawing at room temperature for 2 hours were 3.05×10^4/g and 8×10^4/ml respectively. But after 24 hours the values were 2.7×10^6/g and 7.5×10^8/ml for muscle and thaw drip respectively. This showed that in the beginning the drip had a higher bacterial count and afterwards the TPC of the muscle was found to be greater. Similar trends were observed at other temperature of thawing also.

4.4.5.1.2 Quantitative changes in the bacterial flora of prawn *M. dobsoni* during thawing

The changes in the total bacterial count of prawn *M. dobsoni* frozen as PD at -40°C and stored at -20±2°C for four months are presented. Table 71 represents changes in TPC of skin and muscle of prawn and Table 73, that of drip. Thawing temperatures were +4°C, +15°C and room temperature (29±2°C).

The pattern of changes of the bacterial count in prawn muscle during thawing at different temperatures was very much similar to that in mackerel. In control also, the trend was more or less same except a slight increase at +4°C in bacterial count.

It was observed that thawing at the lowest temperature viz. +4°C caused longer lag period in mackerel and prawn and the rate of multiplication of associated microorganism was also low. According to Stumbo (1947), lower temperatures are required more to arrest the bacterial growth at logarithmic growth phase than to maintain bacteria in the lag phase of growth. Thawing temperature of 4°C, in addition to having a long lag period, possessed lowest multiplication rate of bacteria.

The lag period for frozen prawn and mackerel, varied according to the temperature of thawing, being 8 hours

Table 71

Changes in the TPC of frozen prawn (frozen as PD at -40°C and stored at -20°C for 4 months) during thawing at different temperatures

Thawing time (hours)	Thawing temperature °C	TPC of frozen prawn — Incubation temperature °C			TPC of raw unfrozen prawn — Incubation temperature °C		
		5	RT	37	5	RT	37
0	-	9.1×10^{2}	6.5×10^{3}	6.4×10^{3}	2.8×10^{3}	4.2×10^{4}	1.1×10^{4}
2	4	9.1×10^{2}	6.8×10^{3}	6.6×10^{3}	2.7×10^{3}	4.0×10^{4}	3.8×10^{4}
	15	9.0×10^{2}	6.9×10^{4}	6.2×10^{4}	3.6×10^{3}	4.0×10^{5}	3.1×10^{5}
	RT	2.1×10^{4}	6.6×10^{4}	6.4×10^{4}	7.7×10^{3}	3.1×10^{5}	3.2×10^{5}
9	4[a]	2.0×10^{3}	7.1×10^{3}	7.0×10^{3}	4.1×10^{3}	3.1×10^{4}	3.7×10^{4}
	15	1.0×10^{4}	8.8×10^{5}	7.8×10^{5}	3.8×10^{4}	6.2×10^{6}	3.0×10^{6}
	RT	9.4×10^{4}	1.1×10^{5}	4.2×10^{5}	6.6×10^{4}	1.12×10^{6}	2.1×10^{6}
16	4[b]	9.9×10^{3}	2.1×10^{4}	1.0×10^{4}	8.4×10^{3}	9.8×10^{4}	7.2×10^{4}
	15	6.2×10^{6}	1.0×10^{7}	9.2×10^{7}	5.4×10^{5}	7.2×10^{8}	6.1×10^{8}
	RT	2.1×10^{6}	6.2×10^{7}	6.7×10^{7}	2.6×10^{5}	2.2×10^{8}	3.2×10^{8}
24	4[c]	3.4×10^{4}	9.6×10^{4}	9.2×10^{4}	1.02×10^{4}	2.11×10^{5}	1.2×10^{5}
	15	1.8×10^{8}	3.7×10^{6}	1.75×10^{6}	1.1×10^{5}	2.10×10^{7}	1.9×10^{7}
	RT	8.0×10^{8}	8.2×10^{8}	1.4×10^{9}	6.1×10^{5}	5.8×10^{9}	4.9×10^{9}

a,b and c represent count after 24, 48 and 72 hours of thawing.

3 series of experiments were undertaken. Comparable results were obtained, and typical results are presented here

Table 72

Changes in the total bacterial count in the thaw drip of frozen mackerel (frozen at -40°C and stored at -20±2°C for 4 months) during thawing at different temperatures

Thawing time (hour)	TPC* of thaw drip during thawing at		
	4°C	+15°C	RT 29±2°C
0	-	-	-
6	-	-	-
9	-	-	2.9×10^{3}
16	-	1.8×10^{2}	7.8×10^{6}
18	-	5.4×10^{3}	3.4×10^{9}
24	-	2.8×10^{5}	8.7×10^{9}

*Temperature of incubation is at RT (29±2°C)

Table 73

Changes in the total bacterial count in the thaw drip of frozen prawn (frozen as PD at -40°C and stored at -20±2°C for 4 months) during thawing at different temperatures

Thawing time (hours)	TPC* of thaw drip during thawing at		
	4°C	+15°C	RT 29±2°C
0	-	-	-
2	-	-	8.0×10^{4}
9	-	-	3.1×10^{5}
16	-	-	1.1×10^{7}
18	-	2.4×10^{3}	7.4×10^{8}
24	-	1.8×10^{4}	7.5×10^{8}

*Temperature of incubation is at RT 29±2°C in all cases

at RT, 8-15 at +15°C and one day at +4°C. A lag period of 2 to 3.5 days was observed at a thawing temperature of 5°C for macroni and cheese dinner (Peterson et al., 1962a,b) and one day for macroni and pees (Munce and Buckle, 1981). According to Lawton and Nelson (1954), lag period is not characteristic of the microorganism present, but rather the existing physiological conditions of the frozen product. These authors observed that lag phase in a microorganism could be shortened by increasing the temperature of thawing or the amount of free water of the frozen product.

The mean generation time during thawing at room temperature and +15°C was lower in prawn than in mackerel, being 1.41 and 1.8 at room temperature and 2.64 and 3.63 at +15°C respectively. The lag period was also slightly shorter in prawn. The reason may be that prawn being smaller in size attains environmental temperature more quickly even into the interior part.

For fishes from tropical areas, storage period at ambient temperature varied from 6 hours to 16 hours depending on species (Chinivasagam and Vidanapathirana, 1985) and the TPC at rejection level ranged from 10^6-10^9/g. For prawn P. monodon, TPC at ambient temperature increased from 10^4-10^5 to 10^6-10^9/g after 16 hours at rejection level.

(Reilly et al., 1985). If a bacterial count of 10^7/g is taken as the upper limit of acceptability (Setty et al., 1985), the limit of acceptability for frozen mackerel on the basis of bacterial count, was 24 h, while on the basis of organoleptic scoring (Tables 74 and 75) it was found to be 9-16 hours on thawing at room temperature. This shows that total bacterial count is not a criteria to judge the acceptability of frozen and thawed fish or prawn. In this respect it may be noted that a good correlation existed between the total bacterial count and organoleptic characteristics in the case of ice stored fish and prawn as evidenced from the work of Anand (1976), Surendran (1980), Setty et al. (1985). Damage inflicted during thawing process on the muscle structure may be the reason for the enhanced rate of spoilage of the frozen samples.

4.4.5.2 Qualitative studies

4.4.5.2.1 Changes in the qualitative distribution of bacteria of frozen mackerel during thawing

The changes occurring to the distribution of various microbial genera in the skin and muscle of frozen mackerel after thawing at different temperatures are presented in Table 76. On the onset of thawing at RT (29±2°C), the bacterial flora of frozen mackerel

Table 74

Organoleptic scorings of the frozen mackerel during thawing at room temperature, +15°C and +4°C

Thawing time (hours)	Frozen mackerel thawed at RT	+15°C	+4°C	(Control) unfrozen raw mackerel kept at RT	+15°C	+4°C
0	8	8	8	9	9	9
9	7	8	8	7	8	9
16	4	7	8	4	7	9
24	3	6	8	3	6	8
48	-	4	7	-	5	7
72	-	-	6	-	4	6
96	-	-	4	-	-	5
120	-	-	3	-	-	4

Table 75

Organoleptic scoring of frozen prawn (M. dobsoni) during thawing at room temperature, +15°C and +4°C

Thawing time (hours)	Frozen prawn thawed at RT	+15°C	+4°C	Raw unfrozen (control) prawn kept at RT	+15°C	+4°C
0	8	8	8	9	9	9
9	6	8	8	7	8	9
16	4	7	8	6	7	8
24	3	6	7	4	6	8
48	-	4	6	-	5	7
72	-	-	5	-	4	6
96	-	-	4	-	-	5
120	-	-	-	-	-	4

is very much selected comprising Pseudomonas (14%), Micrococcus (51%), Moraxella (12%), Acinetobacter (14%) and Flavobacterium/cytophaga (7%). At the end of 24 hours of thawing at room temperature, when the fish was organoleptically rejected, the major constituent of the bacterial flora was found to be Pseudomonas (72%). This was followed by Moraxella (10%) and Acinetobacter (11%). On thawing at temperatures +15°C and +4°C for 24 hours, the proportion of Pseudomonas were 76% and 78% and they constituted the bulk of the flora. This showed that the major flora in frozen mackerel at the end of 24 hours of thawing was Pseudomonas and that the effect of thawing temperature on the qualitative composition of the bacterial flora was not very much significant except for the slight increase in the proportion of Pseudomonas at lower temperatures than room temperature.

The bacterial flora of thaw drip of mackerel on thawing at 15°C and room temperature is also presented in Table 76. The bacterial flora contained in the thaw-drip of mackerel was more or less a reflection of the bacterial flora of skin and muscle of frozen mackerel at any temperature studied. But, while the skin and muscle of frozen mackerel showed a prevalance of 30% of Pseudomonas after 9 hours at room temperature, their number in the thaw drip after the same period was

Table 76

Qualitative changes in the bacterial flora* of frozen mackerel R. kanagurta (frozen at -40°C and stored at -20±2°C for 4 months) during thawing at different temperatures

Thawing period hour	Thawing temperature °C	Bacterial species as % of total isolates												Total No. of cultures isolated	
		Pseudomonas		Moraxella		Acinetobacter		Flavobacterium/ Cytophaga		Micrococcus		Others			
		M	D	M	D	M	D	M	D	M	D	M	D	M	D
0	+ 4	20	0	16	0	11	0	6	0	47	0	0	0	63	0
	+15	17	0	16	0	13	0	5	0	49	0	0	0	71	0
	RT	14	0	12	0	14	0	7	0	51	0	2	0	58	0
9	+ 4	40	0	18	0	10	0	11	0	21	0	0	0	68	0
	+15	36	0	15	0	11	0	10	0	28	0	0	0	75	0
	RT	30	60	16	2	16	2	12	0	24	36	2	0	60	68
16	+ 4	67	0	14	0	3	0	2	0	14	0	0	0	53	0
	+15	60	76	17	1	3	0	4	0	16	23	0	0	58	48
	RT	62	72	15	7	3	1	3	0	17	20	0	0	62	58
24	+ 4	78	0	10	0	8	0	4	0	0	-	0	0	57	0
	+15	76	82	8	3	9	5	5	0	2	10	0	0	60	48
	RT	72	80	10	4	11	0	2	0	5	16	0	0	61	55

*Colonies isolated from TGA media; M - Muscle; D- Drip

almost double (60%). These values indicate that in thaw drip there is greater multiplication of the bacteria compared to muscle. Similar behaviour is seen on thawing at +15°C also.

4.4.5.2.2 Changes in the bacterial flora of prawn *M. dobsoni* during thawing

Table 77 represents the changes in the qualitative distribution of major microbial groups in muscle of prawn (*M. dobsoni*) on thawing at room temperature, +15°C and 4°C.

While *Pseudomonas* species emerged as the major flora on rejection of frozen mackerel, prawns harboured *Moraxella* and *Acinetobacter* groups. *Pseudomonas* increased from 14 to 72% in their number while in the case of *Moraxella/Acinetobacter* group it was very less. A lower generation time possessed by *Pseudomonas* at these temperatures compared to *Moraxella* (Lee and Pfeifer, 1975) may be the reason for this type of behaviour.

As far as the bacterial flora is concerned, this study shows that freezing may be regarded as reverse process of thawing. While freezing and frozen storage caused an accumulation of Gram-positives, the reverse namely, the rise of Gram-negatives occurred during thawing. While resistance to cold may be the determining factor

Table 77

Qualitative changes in the bacterial flora* of frozen prawn (frozen as PD at -40°C and stored at -20±2°C for 4 months) during thawing at different temperatures

Thawing period hour	Thawing temperature °C	Bacterial species as % of total isolates												Total No. of cultures isolated	
		Pseudomonas		Moraxella		Acinetobacter		Flavobacterium/ cytophaga		Micrococcus		Others			
		M	D	M	D	M	D	M	D	M	D	M	D	M	D
0	+ 4	8	0	13	0	17	0	3	0	58	0	1	0	60	0
	+15	5	0	12	0	18	0	5	0	60	0	0	0	52	0
	RT	5	0	13	0	15	0	6	0	61	0	0	0	58	0
9	+ 4	8	0	31	0	19	0	2	0	40	0	0	0	53	0
	+15	8	18	28	41	19	20	3	0	41	21	1	0	58	53
	RT	6	20	30	42	15	18	6	0	43	20	0	0	61	61
16	+ 4	7	0	38	0	29	0	6	0	20	0	0	0	57	0
	+15	10	20	35	43	31	26	4	0	20	11	0	0	55	56
	RT	5	16	34	44	30	30	8	0	23	8	0	0	62	59
24	+ 4	10	0	45	0	35	0	6	0	4	0	0	0	53	0
	+15	8	17	44	45	38	28	5	0	5	10	0	0	58	62
	RT	7	14	44	46	36	31	6	0	7	11	0	0	63	60

*Colonies isolated from TGA media; M — Muscle; D - Drip

for the predominance of Gram-positives during frozen storage, the lowered growth rate may the reason for their fall in number during thawing. The inhibitory effect of the component flora (Graves and Frazier, 1963; Peterson et al., 1962) or competition for nutrient supply in mixed cultures (Troller and Frazier, 1963) may be the reason for this decreased growth rate. This is supported by the observation recorded in earlier part of this thesis that Pseudomonas and Micrococcus strains when grown independently in pure cultures(4.3.5) possessed comparable generation times at RT. Hence nature of component flora in mixed population may be the deciding factor.

The present study reveals two major findings of practical significance so far as the thawing process of frozen fish/shrimp is concerned. The species,which were present in low numbers on the onset of thawing, constituted bulk of the total population at the time of spoilage of thawed product. Report similar to this has been stated by Liston and Shewan (1958). Secondly , there is some relation between the initial flora of the raw material before freezing and that at the time of spoilage of fish/shrimp. Moraxella and Acinetobacter species,which formed bulk of the flora of the spoiled prawn,were abundant in raw prawn also as part of native flora. Likewise,Pseudomonas constituted bulk of the flora at

the time of spoilage of frozen and thawed mackerel and these were present in significant numbers in raw mackerel also.

Large number of studies on the spoilage flora of fishes from both tropical and temperate areas consider Pseudomonas as a potent spoiler (Shewan et al., 1960; Lee and Harrison, 1968, Simidu et al., 1969; Gillespie and Macrae, 1975; Surendran, 1980; Barile et al., 1985). But there are reports of Flavobacterium as spoilage flora of fishes at low temperature (Banik et al., 1976). For prawn, alone or together with Pseudomonas, the Achromobacter group was the predominant flora at the time of spoilage at chill temperature (Campbell and Williams, 1952; Cann et al., 1971; Walker et al., 1970; Cann, 1974; Surendran, 1980). Vibrio species also were found to be part of the flora of tropical prawn at the time of spoilage (Chandrasekaran et al., 1985). This study shows that quantitatively as well as qualitatively, spoilage of frozen thawed fish/prawn followed similar pattern of spoilage as that of chill-stored fish or prawn. This is in confirmation to the statement of Hobbs (1982) that spoilage of frozen thawed fish follows the same pattern as that of chill stored fish.

4.4.5.3 Biochemical characteristics of the bacteria isolated from frozen mackerel and prawn during thawing.

Major groups of the bacterial cultures isolated at RT from thawed prawn and mackerel were subjected to various biochemical tests at three temperatures viz. +4°C, +15°C and room temperature (29±2°C). Since the aim of this study was to assess the spoilage potential of the isolates, the behaviour of these cultures in sterile fish press juice was also determined side by side with the biochemical tests. The results are presented in Table 78 and 79.

The biochemical characteristics of the bacterial cultures showed Pseudomonas species to be the most active. Of the total of 126 strains tested at RT, 52% were found to be gelatin liquefiers, 69% produced H_2S and 71% produced acid from glucose. The Micrococcus spp. were biochemically less active than Pseudomonas spp., but more active than Moraxella and Acinetobacter groups. 58% of the total 140 strains liquefied gelatin, 20% hydrolysed casein and 70% produced acid from glucose. Among the Moraxella spp. 20% produced H_2S, 22% liquefied gelatin, 81.8% hydrolysed cascein and 9% produced acid from glucose. 65% of the Acinetobacter spp., produced H_2S, 37.5% liquefied gelatin and 25% hydrolysed casein.

Table 78

Biochemical characteristics of the bacterial strains isolated during thawing at RT of frozen fish/prawn

Tests	Pseudomonas			Moraxella			Acinetobacter			Flavobacterium/ Cytophaga			Micrococcus		
	Temperature of incubation °C														
	4	15	RT	4	15	RT	4	15	RT	4	15	RT	4	15	RT
Production of H_2S	81*	82	87	10	11	11	20	21	21	0	0	0	2	8	11
Production of indole	5	6	6	0	0	0	0	0	0	0	0	0	0	0	0
Gelatin liquefaction	66	64	66	10	12	12	10	12	12	1	4	4	70	78	81
Casein hydrolysis	26	30	36	41	45	45	8	8	8	6	8	8	8	24	28
Phosphatase	50	54	54	0	0	0	0	0	0	2	6	8	2	28	28
D-Nase	22	24	24	0	0	0	12	12	12	0	0	0	3	14	14
Reduction of TMAO	48	56	40	0	0	0	0	0	0	0	0	0	0	0	0
Utilization of glucose. Aerobic	90	90	90	0	0	0	0	0	0	0	0	0	50	70	70
Anerobic	0	0	0	0	0	0	0	0	0	0	4	4	18	28	28
Acid from glucose	117	118	118	0	0	0	0	0	0	2	4	4	68	88	88
Sucrose	117	118	118	0	0	0	0	0	0	2	4	4	68	88	88
Lactose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Maltose	60	70	71	0	0	0	0	0	0	2	4	4	20	22	24
Mannitol	8	10	12	0	0	0	0	0	0	0	0	0	0	0	0
No. of cultures tested	126	126	126	55	55	55	55	32	32	32	12	12	140	140	140

*Number of cultures giving positive reaction

Table 79

Characteristics in fish muscle press juice of the bacterial strains isolated during thawing at RT of frozen stored fish/prawn

		Pseudomonas			Moraxella			Acinetobacter			Flavobacterium / Cytophaga			Micrococcus		
		Temperature of incubation °C														
	Days	4	15	RT	4	15	RT	4	15	RT	4	15	RT	4	15	RT
Production of NH_3	4	0	12+	32	0	0	0	0	0	0	0	0	0	0	0	0
	10	66	97	97	18	23	23	8	14	18	0	0	0	1	6	10
Production of H_2S	4	23	68	68	2	16	8	0	0	0	0	0	0	0	0	0
	10	82	92	92	25	20	21	4	20	23	0	0	2	3	13	14
Production of TMA	4	21	21	23	2	9	6	0	0	0	0	0	0	0	0	0
	10	51	62	67	6	10	10	0	2	2	0	0	0	0	0	0
pH rise	4	10	21	48	0	2	16	0	0	0	0	0	0	0	0	0
	10	100	121	121	42	55	55	4	4	4	0	0	0	8	10	10
Odour	4	16^b	20^b	28^b	0^c	0^c	0^c	0	0	0	0	0	0	0^b	0^b	0^b
	10	100^a	108^a	110^a	20^c	31^c	31^c	0	3	3	1	1	1	4^b	8^b	8^b
TPC/ml	4	10^6	10^5	10^6	10^5	10^6	10^7	10^5	10^6	10^6	10^4	10^6	10^6	10^4	10^6	10^6
	10	10^6	10^9	10^9	10^7	10^8	10^8	10^6	10^8	10^8	10^8	10^7	10^9	10^6	10^7	10^9
Total No. of cultures tested		126	126	126	55	55	55	32	32	32	12	12	12	140	150	140

*Average of the total number of cultures + Number of cultures giving positive reaction
d - strong pungent odour; b-very paint odour; c - after 2 weeks produced mild to strong fruity odour

37.5% of these strains were also deoxyribonuclease positive The Flavobacterium/cytophaga species were also active, 33.3% liquefying gelatin and 66.6% hydrolysing casein.

The behaviour of these isolates in fish press juice was also noted after incubation for 4 and 10 days at the three temperatures viz. +4°C, +15°C and RT. Of the 126 strains of Pseudomonas spp. incubated at RT, 25.4% produced ammonia after 4 days. However, after incubation at this temperature for 10 days, 77% were found to be positive. Incubation of the fish muscle press juice at +15°C for 4 days resulted in the production of ammonia by 9.5% of the strains and 77% after 10 days. At +4°C, the corresponding values were 0 and 52.3%. These results show that there is a striking increase in the number of cultures showing positive reaction with time lapse. The number of cultures showing positive reaction decreased with temperature and a corresponding decrease was noted in TPC also.

The Pseudomonas species were most active both by biochemical tests and behaviour in fish press juice. Majority of them produced slight to strong pungent off odours after keeping for 10 days at RT (62%), +15°C (60%) and +4°C (52%). Of the Micrococcus spp., less than 20% produced very faint odours, after 10 days at RT. None

of them were capable of producing any off odour at +15°C or +4°C. 56.4% of the Moraxella species produced off odour after incubation at RT for 10 days, 10.3% of the Acinetobacter strains and 8.3% of the Flavobacterium strain also did so. On the basis of these results the Pseudomonas species proved to be the most active followed by Moraxella, Micrococcus and Flavobacterium.

As seen from the data the action of the bacterial isolates from fish/prawn was very slow in biochemical tests as well as on fish press juice when incubated at +4°C. The cell number at this temperature was also low, and this was true for all the species tested. A slower growth rate at 8°C of bacterial isolates from tropical fishes compared to those from cold water fishes was noted by Devaraju and Setty (1985). Ingram (1971) reported that amount of chemical change caused by a single cell is so small that alterations detectable by chemical means can be produced only by cell populations reaching maximum numbers. Hence the slow reaction rate at low temperature viz. +4°C can be attributed to the low cell number. This can be the reason why a good proportion of the cultures failed to give positive reaction at low temperature within the specified time. A relation between spoilage and cell number has been noted previously by Liston (1964).

According to this study, the Pseudomonas spp. constituted the active spoilage flora among isolates recovered from frozen thawed fish. Biochemically also they were very active. The Micrococcus, though biochemically more active than Moraxella, were found to be rather inert in fish muscle press juice. On the contrary, the Moraxella spp. though biochemically inactive were able to produce off odours in fish press juice at all temperatures. This is in agreement with the finding of Adams et al. (1964) that biochemical activity is in no way measure of spoilage potential. Many of the microorganisms, which were active biochemically, were found to be non spoilers and vice-versa. Since spoilage under natural condition occur due to the concurrent or successive activities of a variety of microorganisms, the synergistic role played by the non spoilers have to be taken into account for reaching final conclusion.

The Pseudomonas species have been considered as active spoilers in fish Castell et al., 1957; 1959; Shaw and Shewan, 1968; Miller et al., 1972). The role of Alteromonas putrifaciens in spoilage of seafoods is highlighted by the work of Van Spreekens (1977). In shrimp, Achromobacter species were reported as potent spoilers by the work of Walker et al. (1970) and

Hobbs *et al.* (1971). It may be assumed that the same groups which caused spoilage of raw/iced shrimps or fish may be responsible for the spoilage of thawed fish after freezing.

SUMMARY

5. SUMMARY AND CONCLUSIONS

The thesis is mainly concerned with the study of the bacteriology of freezing of mackerel(*Rastrelliger kanagurta*) and prawn (*Metapenaeus dobsoni*)

The thesis in four sections describes the salient features of the observations and inferences on the bacteriology of freezing of fishes and prawns. This includes the evaluation of methodology for the optimum recovery of bacteria, bacteriology of the newly caught fish and prawn, the effect of freezing temperatures on the survival of selected species of isolates from fish and prawn and the bacteriology of freezing, frozen storage and thawing of prawn/fish emphasizing the effect contributed by each.

5.1 Factors affecting the recovery of bacteria

Fresh and frozen fish/prawn showed significant difference in bacterial count between incubation temperatures, RT (29±2°C) giving a higher bacterial count than 37°C or 5°C. Based on the observation that room temperature facilitated growth of all bacteria which could grow at 37°C and 5°C, it was considered that room temperature (29±2°C) would be the most appropriate for explaining the bacteriological aspects of fresh/frozen fish.

Comparison of two methods, spread plate method and pour plate method indicated that spread plate method was giving a higher bacterial count in majority of samples of fresh or frozen fish and prawn. For fresh/frozen fish and prawn, the effect of method was not as significant as temperature.

Effect of some of the commonly used diluents on the relative recovery of bacteria from fresh/frozen fish and prawn showed that in fresh fishes the highest recovery was in Quarter strength Ringer's solution. This was closely followed by n.saline. For frozen fishes, n.saline showed maximum recovery. N.saline and phosphate buffer also produced steady bacterial counts on holding inoculum up to 30 minutes (hold-up time). On the basis of the studies, n.saline was used for further work.

The relationship between total bacterial count and incubation period was also studied and data analysed statistically. For fresh fish, the mean logarithmic increase in count after 24 h was significantly high, whereas in frozen fish the mean logarithmic increase in count after 48 h was significant.

5.2 Bacteriology of newly caught fish/prawn

Quantitative and qualitative nature of the bacterial flora of newly caught mackerel and prawn were investigated

in detail. The average TPC of the skin with muscle, gills and intestine of mackerel at RT (29±2°C) in SWA were $1.2x10^5$/g, $6.4x10^6$/g and $2.4x10^7$/g. The muscle and intestine of fresh prawn had an average TPC of $8.6x10^6$/g and $2.3x10^7$/g at RT (29±2°C) in SWA.

TPC was affected by incubation temperature. Room temperature (29±2°C) gave maximum counts for skin and muscle while the TPC of intestine was slightly high at 37°C. The sea water based agar gave higher count than the distilled water based agar.

The generic distribution of bacteria in the skin with muscle, gills and intestine of mackerel showed that Gram-negative asporogenous rods or coccoids belonging to the genera Vibrio, Pseudomonas, Acinetobacter, Moraxella and Flavobacterium accounted for 89% of the flora. Gills harboured almost similar flora as that of skin with muscle while the intestine harboured slightly different flora.

In freshly caught prawn M. dobsoni, Moraxella and Acinetobacter were the predominating genera followed by Pseudomonas and Vibrio. Major constituents of the intestine were Acinetobacter, Moraxella and Vibrio. The Gram-positives accounted to 25% of the flora.

The incubation temperature showed some influence on

the growth and recovery of different species, so also the medium composition. The study indicated that TPC of the tropical fish and prawn were slightly higher than that reported for cold water types. Qualitatively also, there was difference between tropical prawn/fish and temperate water types. Major difference was in the percentage of Vibrio spp.

5.3 Effect of freezing, frozen storage and thawing on isolated cultures

Selected bacterial cultures were studied for their survival at temperatures of -39±2°C, -20±2°C and +7°C. The general pattern of the survival curve at -39±2°C and -20±2°C showed a steep slope in the first 24 h showing maximum reduction. This was followed by a curve of lesser slope up to 10 days after which it was almost horizontal. At +7°C there was growth and multiplication of all strains expept Vibrio spp. The reduction in bacterial number was calculated as percentage of the initial population. The reduction occurring during intervals of frozen storage was also calculated. These results showed good comparison with the survival curve.

Most of the Gram-negative cultures were sensitive to freezing as well as early storage period. The Gram-positive cocci was less sensitive to freezing period than

frozen storage. Among the cultures Vibrio spp. were most sensitive and Micrococcus spp. least so. Sensitivity to freezing varied among the different strains of the same genera. Death at -20±2°C was slightly lower than that at -39±2°C for most of the strains. The behaviour of pathogens such as Salmonella anatum during freezing and frozen storage was very much identical to that of bacteria isolated from fish i.e. non-pathogens. The study also revealed that even after prolonged storage up to one year at freezing temperatures, complete elimination did not occur in majority of the bacterial cultures.

Methods were standardized for estimation of individual species of bacteria, when present in mixed population. Using these methods, the behaviour of mixed populations of selected bacterial cultures during freezing were followed and data compared with that of the individual specimens. The study showed that Pseudomonas and Micrococcus species behaved identically in mixture as well individual cultures. But the behaviour of Moraxella was slightly different. When present individually, the Moraxella spp. were as sensitive as Pseudomonas spp. But in a mixture of the two, Moraxella seemed less sensitive.

Among the freezing menstrua tried, fish muscle was found to be very protective environment for survival of

these bacterial cultures. The fish muscle exerted its effect during freezing as well as frozen storage. The freezing menstruum, for which lowest survival occurred, varied with different species.

The influence of initial cell number on the survival of selected species showed that population effect existed only at very high concentrations of cells. But a relation could be observed between death rate and cell density in many instances.

The effect of pH on the survival of selected species of bacteria indicated that pH, at which maximum survival occurred, corresponded to the optimum pH of the test strain. Acidic pH was found to be more harmful than alkaline pH.

Studies with cells of different age (phase) showed that cells in the logarithmic phase were more susceptible to freezing temperatures than stationary phase cells. Extended stationary phase had no effect on survival.

Chemicals like sucrose and glycerol protected the bacterial cells. The minimum concentration of substances which effected maximum protection on freezing varied with the species. Salts like sodium chloride and potassium chloride had a harmful effect on survival during freezing. While low levels of sodium chloride was not inhibitory, even very low levels of potassium chloride proved to be lethal.

The metabolic injury measured on minimal agar medium was found to be affected by the nature of suspending fluid. But the temperature between −20°C and −40°C was found to have little effect on the death and injury. Within the given temperature the percentage of dead cells increased with storage while that of injured decreased. Injury and death varied from species to species and was more evident in the freezing period.

The effect of thawing temperatures viz. +2°C, +15°C and RT (29±2°C) on growth and multiplication of frozen cells of selected bacterial species were investigated. All the test cultures showed longest generation times at RT (29±2°C). The growth curve of the frozen cells showed a slight lag in the beginning of thawing. Except that, the growth curve of the frozen cells were comparable to that of unfrozen cells and the growth curve at RT of the frozen cells very much resembled a typical growth curve. Among the test cultures, Pseudomonas possessed greatest growth rate at all the three thawing temperatures.

5.4 Effect of freezing on bacteria present in fish/prawn

The changes occurring quantitatively as well as qualitatively in the bacterial flora of mackerel and prawn were studied in four stages. They were,

1. Preprocess handling
2. Freezing
3. Frozen storage
4. Thawing

Preprocess handling

Washing caused 52 - 98% reduction in the total bacterial count in mackerel, while short time icing had no effect. Qualitatively, the bacterial flora of the iced fish was very much comparable to that of raw fish, except for a slight increase in Pseudomonas spp. Washed fish showed an increase in Micrococcus spp. and decrease of all Gram-negative rods.

For prawn, beheading caused a decrease of 36.21% to 62.01% of TPC. On the otherhand, peeling and deveining caused an increase in TPC by 32.44% to 65.48%.

The beheaded and iced prawn showed similar flora as that of raw material. But peeling and deveining caused an increase in Gram-positives, especially Micrococcus spp. There was also decrease in the Gram-negative rods. Freezing caused significant decrease in the number of bacteria present on skin with muscle and intestine of mackerel. The reduction was 74.25 to 82.29% in block frozen mackerel and 81.4 to 83% in IQF. In prawn, the reduction was 82.98% in whole prawn, 79.48% in headless and 53.1% in PD.

The Gram-negatives, especially the Vibrio sp. showed a drastic fall during frozen storage. There was lesser reduction during frozen storage when compared to the reduction encountered during freezing. But the values were highly variable. Similar trends were observed for both mackerel and prawn.

The effect of freezing was further enhanced during frozen storage. The percentage of Gram-negatives continuously fell while that of Gram-positives increased. Vibrio was most affected and Pseudomonas less so. While Moraxella and Acinetobacter remained almost stationary, the number of Micrococcus strains increased with storage. Compared to mackerel, prawn harboured a greater number of Micrococci. Difference was also observed in the prevalance of Gram-negatives in these two types.

There was wide variations in the occurrence of various physiological groups of bacteria in mackerel and prawn. During freezing and frozen storage, groups such as gelatin liquefiers, hydrogen sulphide producers, indole producers etc. decreased with storage, while the percentage of nitrate producers remained constant. The percentage of acid producers remained stationary during storage of mackerel, while in prawn their number decreased.

Thawing

Quantitative and qualitative changes taking place in the bacterial flora of mackerel and prawn during the process of thawing at different temperatures namely +4°C, +15°C and RT (29±2°C) were investigated. Frozen mackerel and prawn when thawed at the lowest temperature namely +4°C produced lowest TPC. There was a lag in bacterial growth during thawing of frozen samples at RT (29±2°C) and this was less significant at thawing temperatures of +15°C and +4°C. The thaw drip had a higher bacterial count than the muscle in the beginning. But later the muscle showed an increased count than drip. Qualitatively the flora of thaw drip was a reflection of the flora of muscle though higher levels of selective groups were noticed in the drip. The terminal flora in the drip and muscle of mackerel was *Pseudomonas*, while in prawn it was *Moraxella* and *Acinetobacter* groups.

The biochemical characteristics and spoilage potential in fish press juice of the isolated cultures from thawed mackerel and prawn showed *Pseudomonas* to be the most active. *Micrococcus* was biochemically active, but in fish press juice the reactions were poor. The reverse was observed for *Moraxella* spp. The effect of temperature on the biochemical activity was also studied.

The present study is an important investigation in the field of bacteriology of freezing of tropical fish and prawn. In addition to getting some fundamental data on the effect of freezing on bacteria, the study will be useful to commercial sector for implementing quality control monitoring.

This study also points to ample scope for future work. The inactivation occurring to bacterial cells at temperature below minimum growth temperature i.e. around zero has not been investigated and effect produced by prior storage at such temperature may alter the survival during freezing as can happen in the case of chill-stored fish. This study shows that it is an area which requires more detailed studies in future work. The antagonistic or synergistic factors existing among bacterial population may affect the survival while freezing or growth during thawing. Such factors also need more in-depth investigations. Investigations in the above lines may perhaps open up new vistas in the microbiology of tropical fish.

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6. REFERENCES

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LIST OF PUBLICATIONS

1. Effect of incubation period on the plate count of raw, iced and frozen fish.
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