"INCIDENCE OF ENTEROTOXIGENIC *STAPHYLOCOCCUS AUREUS* IN RELATION TO THE MICROBIAL SAFETY OF SEAFOOD"

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DECLARATION

I hereby declare that this thesis is a record of bonafide research carried out by me under the supervision and guidance of Dr. P.K. Surendran, 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.

Place: Cochin

Date: 18.01.07

18/01/07

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This is to certify that this thesis entitled "Incidence of enterotoxigenic *Staphylococcus aureus* in relation to the microbial safety of seafood" embodies the result of original work conducted by Ms. Sindhu O.K, under my supervision and guidance from November 2004 to December 2006. I further certify that no part of this thesis has previously formed the basis for the award to the candidate, of any degree, diploma, associateship, fellowship or other similar titles of this or any other University or Society. She has passed the Ph. D qualifying examination of the Cochin University of Science and Technology, held in November 2005.

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INTRODUCTION

1. INTRODUCTION

Food poisoning is an important problem of public health significance in both developed and developing countries. A number of surveys have shown that consumer awareness about quality of their food is increasing. Millions of people worldwide suffer from disease caused by contaminated foodstuffs. The extensive coverage in the media on food safety issues such as the bovine spongiform encephalopathy (BSE) crisis, use of growth promoters, detection of pesticide, heavy metal and dioxin residues in food, concerns about genetically modified foods, the pathogenic bacterial problem and spread of antibiotic resistant microbial strains add to the consumer fear and unease about what they eat. The situation is further complicated by the fact that many consumers suffer from a serious lack of knowledge on simple food safety issues. A number of socio- economic changes such as increased urbanization; migrations and population demographics are further contributing to the safety of foods. The population of highly susceptible persons is expanding world wide because of ageing, malnutrition, HIV infections and other underlying medical conditions with a weakened immune system.

Seafood is an excellent choice of food, for protein and other essential nutrients. In many regions of the world, seafood and other aquatic products form a major portion of the diet, while in other parts of the world it is viewed as a delicacy. Today more and more people are turning to fish as a healthy alternative to red meat. The low fat content of many fish species and the effects on coronary heart diseases of the n-3 polyunsaturated fatty acids found in fatty pelagic fish species are extremely important aspects for health conscious people, particularly in affluent countries where cardiovascular disease mortality is high (Huss, 1994). Fish and fishery products are in the forefront of food safety and quality improvement, because they are among the most internationally traded food commodities. Besides supporting the health lives of millions of people, seafood also happens to support the growth of pathogenic bacteria, acting as a vehicle for the

transmission of pathogenic organisms that can cause infection or intoxication. So it is responsible for a significant percentage of food borne diseases. The principal biological agents that cause seafood borne disease are bacteria, viruses and parasites. The products of microorganisms such as bacterial toxins, algal toxins or products of bacterial metabolism such as histamine can cause food poisoning when fish or shellfish harboring these products are consumed. The incidence of disease causing agents in seafood has become a reason for the rejection or detention of these products in the international markets.

Seafood differs from other foods in a number of ways. The type of microorganism associated with seafood may vary depending up on whether fresh or processed. Seafood is processed in to a wide range of products and is consumed in many forms like smoked, canned, salted, dried, frozen and raw. Most seafood is harvested from a wild population and the fishermen are hunters with no influence on handling of their prey before it is caught. The seafood processor is limited in his choice of raw material to what is available in respect of size, condition and fish species landed by the fisherman. The microbial flora present on the intestine, surface and gills of fresh fish is an indication of the bacterial flora present in the aquatic environment from where it is caught (Huss, 1994).

The major determinant both in the quality of seafood and safety is the presence of various types of bacteria present in the raw material. Depending on the route of entry in to the fish body microorganisms can be grouped into three classes as bacteria present in the aquatic environment, bacteria derived by way of pollution of aquatic environment and bacteria acquired by seafood from terrestrial environment through contamination (Thampuran, 2003).

The bacterial species that cause human illness encountered in seafood can be classified into three groups (Feldhusen, 2000). These include the members of the family Vibrionaceae, such as *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*,

virulent strains of *Aeromonas hydrophila* and *Clostridium botulinum*, which are indigenous to the environment. Second category comprises of bacteria introduced via faecal contamination, which are mainly members of the family Enterobacteriaceae, such as *Escherichia coli, Salmonella, Shigella* and pathogenic serotypes of *Yersenia enterocolitica*. The third group consists of bacteria introduced while processing and storage such as *Listeria monocytogens*, *Bacillus cereus*, *Clostridium perfringens* and *Staphylococcus aureus*.

Thorough cooking of seafood products would virtually eliminate all microbial and parasitic pathogens. It will not destroy some microbial toxic metabolites. Some of these toxic metabolites are produced by Gram-positive bacterial genera like *Bacillus*, *Clostridium* and *Staphylococcus*. These metabolites show various degrees of heat resistance. Among these toxins Staphylococcal enterotoxin is the most heat resistant one, which is produced by *S.aureus*.

A number of studies have been carried out on the incidence and characterization of *Staphylococcus* isolated from different foods, clinical, bovine and other sources. The present work is an attempt to know the incidence of *Staphylococcus* particularly *Staphylococcus aureus* in various fish and fishery products both meant for internal consumption and export market. The main objectives of the study are:-

- Investigation of the microbial quality of various fish/shellfish and fishery products.
- Study the incidence of *S.aureus* in various fish and fishery products.
- Study the toxigenic potential of *S.aureus* and *Staphylococcus* species from various fish and fishery products.
- Biochemical characterization of *S.aureus* and other *Staphylococcus* species.
- Assess the pathogenic potential of *S.aureus* and other *Staphylococcus* species.
- Assess their antibioic sensitivity.

- Study the effect of environmental conditions on growth and toxin producton by *S.aureus* in *in-vitro* conditions.
- Assess the effect of temperature on growth and toxin production in *in-vivo* conditions.
- Comparison of molecular method and immunological method for the detection of toxigenicity of *Staphylococcus* strains.

About this thesis

In this thesis, the investigation has been dealt in the following way.

A detailed study was made on the microbial quality, with special reference to food safety, of the fish and fishery products in the retail trade in Cochin and around. Also, farmed molluscan shellfishes like mussels and oysters were investigated for the microbial quality including the presence of pathogenic bacteria. Special stress has been given to monitor the incidence of coagulase positive as well as coagulase negative *Staphylococcus* in these products and their relative incidence have been recorded.

In the next part, the investigation was centered mainly on toxigenic *S.aureus*. This is because among the Gram positive toxigenic bacteria, the *S.aureus* with potential to produce thermostable enterotoxins are more relavent in food safety concerning seafoods in comparison with the Gram-negative pathogens like *Salmonella* and *V.cholerae*.

The incidence, toxigenic potential and conditions of toxin production by *S.aureus* have been investigated in detail. An attempt has also been made to relate the toxigenisis with the presence of the concerned toxigenic genes in the genomes of *S.aureus* strains.

The thesis is presented in five chapters. In chapter 1 introduction is given. In chapter 2 a review of literature is presented. A brief review on the quantitative and qualitative aspects of fish bacteriology is given in the initial part. A detailed review on *S.aureus*, their taxonomy status, pathogenicity, detection of toxin production is discussed in detail.

Chapter 3 is the Materials and Methods section. Details of the samples analyzed, media and chemicals used and all the methods employed in the investigation are presented.

In chapter 4, results and discussion are presented. Mostly results are in Tables. Wherever possible, graphical representation of the data is also given. Relevant photographs are also included.

A summary of the work presented in the thesis is given as chapter 5. A detailed bibliography of all the citations made in the thesis is given in the end of the thesis. Also a list of publications by the author is appended.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Bacteriology of fresh fish and shellfish

2.1.1. Bacteriology of fresh fish

Quantitative aspects

The flesh and internal organs of freshly caught healthy fish are normally sterile but bacteria may be found on the skin, gills and in the intestine (Shewan, 1962). The skin of fish usually carries a bacterial load in the order of 10^2-10^7 cfu/cm^2. The gills and intestine also carry a bacterial load of the order 10^3-10^9 /g and 10^3-10^9 /g respectively. This very wide range reflects the effect of environmental factors. The fish taken from temperate waters has counts at the lower end of the range compared with the fish taken from tropical and subtropical waters and polluted areas, which have a higher count (Shewan, 1977). The count in the intestine relates directly to feeding activity, being high in feeding fish and low in non-feeding fish (Liston, 1956). The North Sea fish has a bacterial load of 10^2-10^5/cm^2 of skin, 10^3-10^7/ g of gills and 10^3-10^8/g of intestinal contents (Shewan,1962 ; Georgala,1958; Liston,1956)while for a tropical fish a bacterial load of 10^3-10^7/cm^2 of skin, 10^5-10^8/g of gills and 10^5-10^9/g of intestine have been reported (Karthiyani and Iyer,1967; 1971). Surendran (1980) recorded a bacterial load of 10^3-10^5/cm^2 of skin, 10^3-10^5/g of gills and 10^3-10^7/g of intestine of the Indian mackerel. The viable bacterial counts on the skin and slime, gills and intestine of fish caught in a particular area showed variations and it reflected similar variations in the environment. Liston, (1956; 1957) observed two peak loads at 0°C and 20°C in the flora of slime and gills of sole and skate in the late spring and autumn. Seasonal variation in the count varies with the species of the fish. Surendran (1980) reported that high bacterial counts of skin, gills and intestine of oil sardine are obtained during June and September- October season, March, October- November season, February- march season and September-December

season respectively. Similarly for Indian mackerel, the highest counts in skin, gills and intestines were reported during January –February season and March, September-December seasons respectively.

Most of the studies reported a total bacterial count in the range of 10^4-10^6cfu/g in fresh fish and shellfish. The microbial quality of retail fish fillets in the Netherlands had bacterial counts of 10^6cfu/g (Vanden Broek *et al,* 1984). Similar results were reported by Chang *et al* (1984) in *Sardinops melanostica* from Korea and Torres- Vitela *et al* (1997) from Mexico.

The study carried out by many workers from Asian countries reported similar results (Nambiar and Iyer 1990; Lakshmanan *et al* 1984; Thampuran and Gopakumar 1990; Karunasagar *et al* 1992; Abraham *et al* 1992; Anand *et al* 2002 and Surendraraj *et al* 2005). In most of these studies, a bacterial count in the range of 10^4-10^8cfu/g with an average count of 10^6cfu/g have been reported.

Qualitative aspects

Several investigators have concluded that the microorganisms associated with most fishery products reflect the microbial population in their aquatic environment (Liston 1980; Colby *et al* 1993; Ashie *et al* 1996; Gram and Huss 1996). Variations in marine environment affect the type of bacteria present on the skin and gills and surface of newly caught fish. Thus, in the cooler seas of the Western hemisphere, bacterial flora is dominated by psychotropic Gram-negative rods. Liston (1957) reported that flora of North Sea flat fish (skate and lemonsole) comprised of *Pseudomonas species* (60%) *Acinetobactor/Moraxella* (14%), with the majority of the remainder being other types of Gram –ve rods. Georgala (1958) found that bacterial flora of the North Sea cod consisted of *Pseudomonas* (44%) and *Acinetobacter* (32%) together with a variety of miscellaneous types.

In the warmer waters of India, east coast of South Africa, Australia and in the Adriatic Sea, flora was dominated by mesophiles, among which *Micrococcus* was the most important species (Wood, 1953; Gillspie and Macrae 1975). The qualitative composition of fish from temperate waters mainly comprised of the genera *Pseudomonas, Moraxella, Acinetobactor, Flavobacterium, Cytophaga* and *Vibrio*, which constituted about 80% of the population (Liston 1980).

Venkataraman and Sreenivasan (1952, 1955) reported the presence of Gram-positives and very low percentage of *Acinetobactor, Moraxella, Flavobacterium* and *Vibrio* as flora in 5 out of 7 tropical fish samples. However, recent studies indicate that the flora of tropical fish is very similar to that of cold-water species. The flora on tropical fish often carries a slightly higher load of Gram-positive bacteria compared with fish from colder waters (Liston, 1980). Studies by Karthiyani and Iyer (1975); Surendran (1980); Surendran and Gopakumar (1983); Surendran *et al* (1989); Abraham *et al* (1992) and Shetty and Shetty (1990) indicated the predominance of Gram negative asporogenous rods like *Vibrio, Pseudomonas, Acinetobactor, Flavobacterium, Cytophaga, Aeromonas* etc in tropical fishes like oil sardine, Indian mackeral and Jew fish.

2.1.2 Bacteriology of fresh crustacean shellfish

Green (1949) found that whole shrimp examined after employing trawl net varied in bacterial counts from 10^3-10^6cfu/g. Bacterial counts of shrimps caught in commercial fishing nets in widely scattered areas in the open Gulf of Mexico, off the Lousiana coast was found to be $4.2x10^4$cfu/g. Cann (1977) showed that figures on total viable counts on temperate and cold water crustaceans ranged between 10^3-10^7cfu/g.

Surendran *et al* (1985) reported the total plate count of *P.indicus, M.affinis* and *M.dobsoni* at 28 ± 2°C in the range 10^5, 10^2-10^6/g and 10^3-10^6/ g respectively. Similar results were obtained by many authors from India (Lakshmanan *et al* 1984; Krishnamurthy and Karunasagar 1986; Karunasagar *et al* 1992; Thampuran and

Gopakumar 1990; Anand *et al* 2002) and other Asian countries like Malaysia (Cann 1977); Sri Lanka (Sumner *et al* 1982; Fonseka and Rana 1990; Jayaweera and Subhasinghe 1990), Taiwan (Chen *et al* 1991) and Pakistan (Zuberi and Quadri 1992).

In general it can be seen that irrespective of the water bodies, the bacterial load in shrimps varied between 10^3-10^7/g.

Qualitative aspects

The main groups of bacteria comprising the flora of crustacean shellfish are *Micrococcus, Coryneforms, Achromobactor* and *Pseudomonas* together with fewer numbers of *Flavobacterium/ Cytophaga* and *Bacillus* (Cann, 1977). Their proportion in composition varies with the temperature of waters in which the animals live, cold-water species having largely *Pseudomonas* and *Achromobacter* (Liston, 1980), while *Micrococcus* and *Coryneforms* dominate in crustaceans from warm waters (Sreenivasan, 1959). Gulf shrimp contained largely *Achromobactor, Micrococcus*, *Pseudomonas* and *Bacillus* (Williams and Ree, 1952 ; Williams *et al* 1952). *Micrococcus* dominated in the flora of Lousiana crab (Alford *et al,* 1941) while *Achromobactor* dominated in Pacific crab (Lee and Pfeifer, 1975). Gram negative flora dominated in shrimps from Cochin (Karthiyani and Iyer, 1975; Surendran *et al,* 1985;) while the dominance of Gram positive bacteria was noticed in shrimps collected directly at sea Off Mangalore (Krishnamurthy and Karunasagar 1986).

2.1.3. Bacteriology of Molluscan shellfish

Molluscan shellfish, except a few true marine species grow and reproduce in and are harvested from estuarine areas. The quality of shellfish depends on the quality of overlying estuarine waters. The bacterial counts in molluscan shellfish were in the range of 10^3 to 10^8/cm^2 (Cann, 1977). Oysters were found to harbour a bacterial load in the range of 10^3 to 10^6cfu/g (Colwell and Liston, 1960; Durairaj *et al,* 1983).

Bacterial counts of clams were found to vary in different studies. A count of 10^4/g was detected in Chesapeake Bay soft shell clam (*Mya arenaria*) (Chai *et al* 1990), 10^7/g in West African clam (*Egeria radiata* Lamarch) (Ekanem and Adegoke1995) and 10^5 to 10^6/g in black clam (*Villoritta cyprinoides*) (Vijayan *et al,* 1982; Balachandran and Surendran, 1984; George and Gopakumar, 1995).

The average bacterial counts of mussel (*Perna viridis*) from Calicut were in the range of 10^3 to 10^6 /g (Surendran *et al,* 1986).

In conclusion the bacterial load of molluscan shellfish is a reflection of the aquatic environment from where it is harvested. The shellfish samples collected from polluted waters especially from faecal contaminated waters generally harbour high bacterial load and pathogens in their meat due to its filter feeding nature.

Qualitative aspects

Flora of molluscan shellfish like oysters were mainly composed of Gram-negative bacteria like *Achromobacter, Pseudomonas, Flavobacterium* and *Micrococcus* (Thanikawa, 1937; Colwell and Liston, 1960; Lovelace, 1968 and Durairaj *et al,* 1983). The main groups of bacteria constituting the flora of clams are Gram-negatives (78%) composed of *Vibrio, Aeromonas, Pseudomonas, Shewanella, Moraxella, Acinetobactor,* Enterobacteriaceae and a smaller portion by Gram positives like *Bacillus, Micrococcus, Arthrobactor, Chromobacterium* and *Staphylococcus* (Lalitha and Surendran, 2005). *E.coli* and faecal coliforms were detected from Chesapeake Bay soft shell clam (*Mya arenaria*) (Chai *et al,* 1990) and West African clam (*Egeria radiata*) (Ekanem and Adigoke, 1995). In addition, faecal streptococci also were detected in black clam (*Villoritta cyprinoides*) (Vijayan *et al,* 1982; Balachandran and Surendran, 1984; George and Gopakumar, 1995). The presence of *E.coli*, coliforms and faecal streptococci in the molluscan shellfish samples indicates the faecal contamination of the aquatic environment due to human interventions.

2.1.4. Bacteriology of fresh water fish

Quantitative aspects

Fresh water fish tend to have lower counts than marine species viz, 102 to 105cm-2 for skin and 104 to 107/g for gut content of temperate fish and 103 to 105 cm-2 of skin surface and 104- 106/g guts of tropical species (Shewan, 1977). Similar figures were reported for catla (*Catla catla*), rohu (*Labeo rohitha*), mrigal (*Cirrihinus mrigala*) and calbasu (*Labeo calbasu*) from India (Surendran and Gopakumar, 1991; Joseph *et al,* 1988). A slightly high count of 106/g were reported from fresh yellow perch (*Perca flavascens*) (Kazanas, 1968).

Qualitative aspects

Studies carried out by Kazanas (1968) in fresh yellow perch (*Perca flavascens*) showed the presence of following bacteria, viz; *Flavobacterium, Micrococcus, Sarcina, Achromobacter, Alcaligens, Pseudomonas, Microbacterium, Vibrio, Bacillus, Corynebacterium, Lactobacillus, Brevibacterium* and *Aeromonas*. Sen *et al,* (1968) could isolate *Micrococcus, Klebsiella* and Enterobacteriaceae from the surface of *Cyprinus carpio var.communis*. Surendran and Gopakumar, (1991) carried out the microbiological examination of the fresh water fishes rohu (*Labeo rohitha*), calbasu (*Labeo calbasu*), mrigal (*Cirrihinus mrigala*), and Catla (*Catla catla*). They reported the presence of *Pseudomonas, Moraxella, Acinetobactor, Vibrio, Micrococcus, Arthrobactor, Lactobacillus, Bacillus* and Enterobacteriaceae in these species. Lilabati and Viswanath, (1998) could isolate *Acinetobacter, Aeromonas, Flavobacterium, Micrococcus, Moraxella, Pseudomonas and Vibrio* from the gills, intestine, skin and muscle of the iced fresh water fish (*Notopterus chitala*) sold in the retail markets of Imphal (Manipur).

2.1.5. Bacteriology of Frozen fish and shellfish

Quantitative aspects

The microbiological quality of frozen fish depended upon various factors such as the nature of the raw material, its pre and post harvest treatments and the sanitary

condition of the processing factories, the rate and nature of freezing, the temperature and length of storage, the original numbers, growth during storage, thawing process and physical protection offered by the food (Luyet and Gebenio, 1940; Larkin *et al,* 1955 Keroluk and Gunderson, 1959)

The bacterial load of frozen fish, shellfish and fish products found to vary between 10^2 to 10^6cfu/g. A bacterial load of 10^2 to 10^6cfu/g in frozen fish (Thampuran *et al,* 1981; Iyer and Shrivastava, 1989 b; Nambiar and Iyer, 1990; Lakshmanan *et al,* 1991; Bandekar *et al,* 2003; Gnanambal and Patterson, 2005), frozen shrimp (Swartzentruber *et al,* 1980; Varma *et al,* 1985; Iyer and Shrivastava, 1989 b ; Aravindan and Sheeja, 2000) and battered fish and shellfish products (Surkiewicz *et al,* 1967,1968; Baer *et al,* 1976) have been reported. The bacterial load in frozen products is an indication of the initial bacterial load in the product before freezing, hygiene of processing plant, effectiveness of freezing, post process handling and time temperature abuse during storage and transportation.

Qualitative aspects

The effect of freezing on the microbiological population is dependent on several factors. There is an initial mortality attendant on the freezing process itself. This varies with the type of organism. The initial reduction in number of bacteria immediately after freezing can range from only one or two percent up to 90% (Shewan, 1961; Simmonds and Lamprecht, 1980). Gram-negative organisms are generally more sensitive to freezing than Gram-positive organisms while spores and food poisoning toxins are unaffected by freezing (Shewan, 1961). The response to freezing varies greatly from strain to strain. *Pseudomonas* species are susceptible. *Salmonella* and *Enterobacteriaceae* are among the more sensitive types (Raj and Liston, 1961). The cocci including marine micrococci and enterococci are more resistant to freezing. The coliform count especially that of *E.coli* on frozen prawns have been found to drop by 95% or more during freezing and frozen storage (Lekshmy, 1964) while faecal streptococci were relatively unaffected. For this

reason, faecal streptococci had been preferred as indicators of factory hygiene. Indicator bacteria like *E.coli* and *S.aureus* were isolated from many frozen fish and fish products (Raj and Liston, 1961; Surkiewicz *et al,* 1968; Baer *et al,* 1976; Swartzentruber *et al,* 1980). The presence of pathogens like *Salmonella* and *Vibrio cholerae* non-O1 were reported from different products (Varma *et al,* 1989; Iyer and Shrivastava, 1989a; Lakshmanan *et al,* 1993; Aravindan and Sheeja, 2000; Gnanambal and Peterson, 2005).

2.1.6. Bacteriology of dried fish and shellfish

Quantitative aspects

Bacterial counts on fully cured seafood are generally low, unless there has been extensive surface contamination. Only halophilic bacteria that have no public health significance can grow on such foods. The Gram-negative bacteria, which are predominant in fresh fish, are relatively sensitive to high salt concentrations and so bacterial numbers decline. Most of the studies reported a bacterial count in the range of 10^3 to 10^5 in different cured dried fish and shellfish (Sreenivasan and Venkataraman, 1958; Solanki and Sankar, 1986, Basu *et al,* 1989). A slightly higher count in the range of 10^3 to 10^6 have been reported in cured fish and shrimp from different markets (Joseph *et al,* 1983; Valsan *et al,* 1985; Joseph *et al,* 1986).

Qualitative aspects

The bacteria represented by the genera *Halobacterium* and *Halococcus* were isolated from cured products (Shewan, 1971). Velanker, (1952) reported the predominance of spore- bearing rods in cured fishes from India. Study carried out by Sreenivasan and Venkataraman, (1958) reported the presence of *Bacillus, Micrococcus, Coryneforms, Achromobactor* and *Flavobacteria.* The presence of indicator bacteria like *E.coli,* faecal streptococci and *S.aureus* were reported in few cases (Valsan *et al,* 1985; Joseph *et al,* 1986). None of the studies reported the presence of any other bacteria of public health significance from these products.

2.2. Bacteria of public health significance from fish and shellfish

Fish and shellfish are occasionally found to be the source of food poisoning caused by pathogenic bacteria. The pathogens are generally derived from three sources Viz: (a) agents naturally present in the environment, (b) derived from pollution and (c) acquired by the fish by way of handling, processing and marketing (Thampuran, 2003). The pathogens in seafood that are naturally present are the members of the family *Vibrionaceae, Aeromonadaceae* and the species *Clostridium botulinum,* particularly type E. The water temperature naturally has a selective effect on the pathogens present. Thus the psychrophilic organisms like *Clostridium botulinum* and *Listeria monocytogens* are common in Arctic and other colder climates, while the mesophilic types *Vibrio cholerae* and *Vibrio parahaemolyticus* are part of natural flora on fish from coastal and estuarine environment of temperate and warm tropical zones (Huss, 1994).

2.2.1. Seafood borne pathogenic bacteria

2.2.1.1. Non-Indigenous bacteria

2.2.1.1.1 *Escherichia coli*

The natural habitat of *Escherichia coli* is the intestine of human and vertebrate animals. Generally the *E.coli* strains that colonize the gastrointestinal tract are harmless commensals (Huss, 1994). But some of the *E.coli* strains particularly those belonging to the classes of enteropathogenic *E.coli* (EPHC), enteroinvasive *E.coli* (EIEC), enterotoxigenic *E.coli* (ETEC) and enterohemorrhagic *E.coli* (EHEC) are causing diseases (Doyle, 1989).

E.coli may be isolated from environments polluted by faecal material or sewage. The organism can survive and even multiply in warm tropical waters (Rhodes and Kator, 1988; Jimnez *et al,* 1989). In temperate waters, this organism is absent from fish and crustaceans at the time of capture. The organism is particularly useful as indicator of contamination (small numbers) or mishandling such as temperature abuse in product

handling (large numbers). Contamination of food with *E.coli* implies a risk that one or more enteric pathogens may have gained access to the food. However, failure to detect *E.coli* does not ensure the absence of enteric pathogens (Silliker and Gabis, 1976). It is also a recognized indicator of possible contamination with enteric pathogens (Bonnel, 1994; Liston, 1980; D'Aoust, 1989). The resistance of *E.coli* to adverse physical and chemical conditions is low. There is no good correlation between *E.coli* and enteric pathogens because it dies off faster than the latter (Temple *et al,* 1980; Burton *et al,* 1987). This makes *E.coli* less useful as indicator organism in examination of water and frozen or otherwise preserved fish products.

The incidence of *E.coli* in fresh finfish has been reported by many workers (Lakshmanan *et al,* 1984; Anand *et al,* 2002; Rao and Gupta, 1978). Anand *et al,* (2002) reported an *E.coli* count in the range of 0-100cfu/g in fresh finfishes landed in Tuticorin fishing harbour.

E.coli is commonly found in different seafood's like scallops, mussels and clams (Vijayan *et al,* 1982; Gorczyca *et al,* 1985). Their presence in fresh and frozen shrimps from Asian countries has been reported (Varma *et al,* 1985; Lekshmy and Pillai, 1964a,b; Sumner *et al,* 1982; Jayaweera and Subashinghe, 1990; Jeyasekaran *et al,* 1990; Anand *et al,* 2002). The prawn from deeper waters showed the absence of *E.coli* (Fonseka and Rana, 1990; Cann, 1977). Contrary to this result, Rao and Surendran, (2003) reported the presence of *E.coli* in deep-sea fish (*Psenopsis cynea*) and shrimp (*Heterocarpus woodmasoni*). They also reported the high prevalence of *E.coli* in fish collected from the retail markets of Cochin.

Since this organism is present in the intestine of human and other warm-blooded animals, it can be concluded that the presence of *E.coli* in different fish, shellfish and fishery products indicates faecal contamination.

2.2.1.1.2. *Salmonella*

Salmonella are members of the family Enterobacteriaceae and occur in more than 2000 serovars. These mesophilic organisms are distributed geographically all over the world. The principal symptoms of Salmonellosis (non-typical infections) caused by *Salmonella* are non-bloody diarrhoea, abdominal pain, fever, nausea and vomiting which generally appear 12-36 hours after ingestion. However, symptoms may vary considerably from grave typhoid like illness to assymptomatic infection. The infective dose in healthy people varies according to serovars, foods involved and susceptibility of the individual. There is evidence for a minimum infection dose (M.I.D) of as little as 20 cells while other studies have consistently indicated a higher M.I.D (Varnam and Evans, 1991).

The natural habitat of *Salmonella* is the gastrointestinal tract of mammals, birds, and reptiles (Pelzer, 1989), but principally occurring in the gut of man and animals and in environments polluted with human or animal excreta. Open marine waters are free from *Salmonella,* but estuaries and contaminated coastal waters may harbour the pathogen. Also, poor personal hygiene may transmit this organism. *Salmonella* species reach aquatic environment through faecal contamination. This accounts for the occasional detection of *Salmonella* from fish and fishery products.

The incidence of *Salmonella* in fish and fish products has been reported by many investigators (Shewan, 1962; Bryan, 1973; Cann, 1977; Anderson *et al,* 1971). Their presence in tropical finfish has been reported by (Nambiar and Iyer, 1990; Iyer, 1985; Iyer *et al,* 1986; Nambiar and Surendran, 2003 a, b; Kumar *et al,* 2003). The incidence of *Salmonella* in different studies varied between 5.76% in fresh fish from retail markets of Cochin to 30% in finfishes from the markets of Mangalore.

Most of the studies revealed the presence of *Salmonella* in fresh and frozen fish and shellfish (Varma *et al,* 1985; Venkateswaran *et al,* 1985; Iyer and Shrivastava, 1989a; Prasad and Rao, 1995; Hatha and Lakshmanaperumalsamy, 1997; Nambiar and

Surendran, 2003a). The study carried out by Anderson *et al,* (1971) in U.S and Pongpen *et al,* (1990) from Thailand reported the presence of *Salmonella* in cooked frozen shrimps.

Salmonella has been isolated from freshwater fish culture ponds in many countries. A survey in Japan showed that *Salmonella* are present in 21% of eel culture ponds (Saheki *et al,* 1989). The incidence of *Salmonella* in cultured fresh water catfish in the USA was estimated to be 5% (Wyatt *et al,* 1979).

The presence of *Salmonella* in fresh and frozen molluscan shellfish has been reported in many studies. Brands *et al,* (2005) reported the prevalence of *Salmonella* in 7.4% of the oysters isolated from United States. It has been isolated from green mussels (*Perna viridis*) (Gore *et al,* 1992;) and clam meat (Kumar *et al,* 2003).

It is concluded that *Salmonella* is not present in the marine waters naturally. The presence of *Salmonella* in fish and fishery products indicates the contamination that has occurred during handling, transportation or processing. The main source of *Salmonella* in fish and shellfishes is water from culture ponds, coastal seawater, process water, ice, shrimp contact surfaces, floor and rodent and lizard droppings.

2.2.1.2. Indigenous bacteria

2.2.1.2.1. *Vibrio* species

The genus *Vibrio* contains a number of species, which are pathogenic to man.The pathogenic species are mostly mesophilic, ubiquitous in tropical waters and in high numbers in temperate waters during late summer or early fall. The disease associated with Vibrios is characterized by gastro enteric symptoms, varying from mild diarrhoea to the classical cholera with profuse watery diarrhoea. One exception is infection with *Vibrio vulnificus*, which are primarily characterized by septicemia. Most Vibrios produce powerful enterotoxins. As little as 5 micro gram cholera toxin (CT) administered orally caused diarrhoea in human volunteers (Varnam and Evans, 1991). A number of other

toxins also are produced by Vibrios. Though named pathogenic, *Vibrio* species are not always pathogenic. The majority of environmental strains lack the necessary colonization factors for adherence and penetration, appropriate toxins or other virulence determinants necessary to cause disease. Vibrios are frequently isolated from seafood and in particular from shellfish.

2.2.1.2.1.1. *Vibrio cholerae*

It is the etiological agent of cholera, a devastating disease that causes severe dehydrating diarrhea and death in healthy people. *Vibrio cholerae* occurs in two serotypes, the O1 and non-O1. The O1 serotype occurs in two biovars, the Classical and El –tor. The Classical biovar serovar- O1 is restricted to parts of Asia (Bangladesh). The El-tor biovar causes most of the cholera outbreaks. The organism may be transmitted from the environment to humans by a number of routes including consumption of under-cooked seafood or shellfish.

Fish and other seafood have been implicated as a source of cholera outbreaks (Joseph *et al,* 1965). This organism gain access to these products, either from the aquatic environments or through different stages of processing. Their prevalence in different aquatic environments is well documented. It has been isolated from brackish water (Lee *et al* 1982; Blake *et al* 1980), marine water (Kenyon *et al* 1984; Mathew 1986), fresh water (Blake *et al,* 1980). Their presence in these water bodies indicates the autochthonous nature of these organisms.

V.cholerae non-O1 is a less virulent strain of *V.cholerae,* which lacks the genetic potential for causing epidemic diseases. It is more prevalent in seafoods than *V.cholerae* O1. Different studies revealed the possibility of contamination of fishery products with *V.cholerae* non- O1 during different stages of handling and processing (Delacruz *et al,* 1990; Iyer and Varma, 1990b; Iyer *et al,* 1990b).

Many studies reported the incidence of *V.cholerae* in fresh and frozen seafood (Berry *et al,* 1994; Wong *et al,* 1995; Baffone *et al,* 2000; Elhadi *et al,* 2004 and Varma *et al,* 1986).

Comparing with finfish, the incidence of *V.cholerae* in shellfish is high. This can be attributed to their chitinase activity, which plays an important role in this association. The chitinase activity may increase the affinity of Vibrios to crustaceans. This explains why *V.cholerae,* a chitin digester, is found more frequently in crustacean shellfishes like shrimps and crabs than other seafood.

The incidence of *V.cholerae* in molluscan shellfish like oysters and clams has been reported (Twedt *et al,* 1981; Wallace *et al,* 1999; Namdari *et al,* 2000). Molluscan shellfishes are generally filter feeders. The presence of *Vibrio cholerae* in these products might be due to the uptake and accumulation in the gastro intestinal tract of these organisms.

It can be concluded that the presence of *V.cholerae* in fish and fish products are inevitable. Only way out is the hygienic handling, processing and proper cooking of fish and fishery products.

2.2.1.2.1.2. *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is a halophilic bacterium that occurs world wide in estuarine environment (Joseph *et al,* 1983; Karunasagar *et al,* 1986). Studies reveal that thermostable direct haemolysin (TDH) and the related haemolysin (TRH) encoded by the *tdh* and *trh* genes respectively are the major virulent factors of this organism (Nishibuchi and Kaper, 1995; Okuda *et al,* 1997a). It can cause gastroenteritis in humans and the illness is most frequently associated with the consumption of raw or undercooked seafood and seafood contaminated with bacterium after cooking (Rippey 1994). It has been isolated from a high percentage of fresh and refrigerated seafood (Wong *et al,* 1992) and frozen seafood (Wong *et al* 1995). This organism has been recognized as an important

cause of food borne illness from consuming raw or undercooked seafood in Asia and North America (Bag *et al,* 1999; De Paola *et al,* 2000; Matsumoto *et al,* 2000; Okuda *et al,* 1997a). It has been revealed that there is a high percentage of incidences of *V.parahaemolyticus* in seafood imported from Asian countries (Wong *et al,* 1999).

The incidence of *V.parahaemolyticus* was reported from crustacean and molluscan shellfish (Colakoglu *et al,* 2006; Ottaviani *et al,* 2005; Brenton *et al,* 2001; Chang *et al,* 1996; Broek *et al,* 1979). Even though this organism is considered as halophilic in nature, its seasonal distribution in fresh water environments and association with fresh water fishes were reported by Sarkar *et al,* (1985) from Calcutta. Many studies from India reported the isolation of these organisms from different fresh and frozen fish and shellfish (Chatterjee, 1980; Bandekar *et al,* 1982; Pradeep and Lakshmanaperumalsamy, 1986; Karunasagar *et al,* 1990; Thampuran, 1996; Sanjeev, 1999; Sanjeev *et al,* 2000 and Prasad and Rao, 1994).

V. parahaemolyticus is a halophilic pathogen, which becomes a health hazard only under certain conditions like raw consumption of seafood or recontamination of cooked fish and fish products.

2.2.1.2.2. *Clostridium botulinum*

Clostridium botulinum is widely distributed in soil, aquatic sediments and fish (Huss, 1994; Huss and Pedersen, 1979). It is also found in sewage, rivers, lakes, seawater, fresh meat and fish (Haagsma, 1991). Human botulism is a serious but relatively rare disease. The disease is an intoxication caused by the *C.botulinum* toxin pre-formed in food. Symptom may include nausea and vomiting followed by a number of neurological signs and symptoms, visual impairment (blurred vision), loss of normal mouth and throat functions, weakness of limbs or total paralysis and respiratory failure which is usually the cause of death (Huss, 1994). The majority of the food borne botulism associated with fish in the Northern and temperate regions are associated with *C.botulinum* type E, where as

in tropical area *C.botulinum* type C and D predominate (Huss, 1981; Dodds 1993a, b). Sometimes fish and fish products have been a vehicle for type A and B botulism. The food poisoning caused by *C.botulinum* type F by the consumption of herring was reported from Czech Republic (Sramova and Benes, 1998). The severity of botulism makes *C.botulinum* an important food borne pathogen. World wide approximately 450 outbreaks with 930 incidents are recorded annually, 12% of which are caused by psychrotrophic serotype of *C.botulinum* type E (Hatheway, 1995).

C.botulinum becomes a hazard when processing practices are insufficient to eliminate the *C.botulinum* spores from raw fish, particularly because of improper thermal processing (Chattopadhyay, 2000). The growth of *C.botulinum* and toxin production depend on appropriate conditions in food before eating, the temperature, oxygen, pH, water activity and the presence of preservatives and competing microflora (Johnson, 2000). Majority of the fish botulism are associated with fermented fish (Huss, 1981). Botulinum toxin formed in the raw material will be found again or even increase in situ in the final products such as heavily salted, marinated or fermented fish (Huss and Rey Petersen, 1980).

The prevalence of *Clostridium* particularly non-proteolytic type E in different fish and shellfish samples are extensively studied and reported (Christiansen *et al,* 1968; Hielm *et al,* 1998; Hyytia *et al*, 1999; Korkeala *et al,* 1998).

In India, *Clostridium botulinum* has been reported from fish and environments (Lalitha and Iyer, 1990; Lalitha and Surendran, 2002).

2.2.1.2.3. *Listeria monocytogens*

Listeria monocytogens is a Gram-positive motile bacterium that grows well at 37°C but which at the same time is psychrotolerant and halotolerant. Seven species of *Listeria* are known and of these, only *L.monocytogens* is pathogenic to humans (Farber & Peterkin, 2000). *L.monocytogens* is an organism indigenous to the general environment

where it is typical of decaying plant material. In addition, it occurs in the gastrointestinal tract of 2-6% of humans, who are healthy carriers. The organism has not been isolated either from free open waters or from fish caught or cultured in such waters. In contrast, water close to agricultural run-off harbour the organism and in principle, the bacterium must be assumed present albeit in low levels on raw fish (Gram, 2001, Huss *et al,* 1995).

Many studies reported the incidence of *Listeria monocytogenes* in different fish shellfish and fishery products (Lennon *et al,* 1984; Weagent *et al,* 1988; Boerlin *et al,* 1997; Gram, 2001; Fuchs and Reilly, 1992; Fuchs and Surendran, 1989; Facinelli *et al,* 1989; Frederiksen, 1991). Review on incidence of *Listeria* in seafood world wide (Embarek, 1994) found the prevalence of *Listeria monocytogenes* varied from 4-12% in temperate areas and the low prevalence of (0-2%) in tropical waters. The presence of this organism in processing environment also has been reported (Autio *et al*, 1999; Vogel *et al,* 2001).

2.3. Enterotoxigenic *Staphylococcus aureus* in fish and shellfish

2.3.1. *Staphylococcus aures* - Historical

The genus *Staphylococcus* is included in Sec.12 Vol (2) of the Bergey's Manual of Systematic Bacteriology (Sneath *et al*, 1986).

Kingdom	: Prokaryotae
Division	: Firmicutes
Class	: Firmibacteria
Family	: Micrococcaceae
Genus	: *Staphylococcus*

Currently 38 species of *Staphylococcus* is recognized (Shale *et al*, 2005*). S.aureus* is the most common species in the *Staphylococcus* genus. It is a bacterial species of great concern in food industry.

Scottish surgeon Sir Alaxander Ogston in 1880 published the data relating the existence of a cluster forming coccus as a causative agent of a number of pyogenic diseases in man. Subsequently in 1882 he named the organism as *Staphylococcus* a name derived from the Greek 'Staphylae' meaning a 'bunch of grapes' and 'coccus' meaning a 'grain or berry'. Rosenbach in 1884 was the first person to grow Staphylococci in pure culture and to study their characteristics in the laboratory. The first report associated with Staphylococci was by Vaugham and Sternberg (Dack, 1956) who in 1884 described an investigation of a large outbreak of illness in Michigan believed to have been caused by eating cheese that was contaminated with Staphylococci. In 1914, Baber clearly demonstrated that Staphylococci were able to cause food poisoning, as the result of the consumption of milk from a cow suffering from staphylococcal mastitis. Dack was able to demonstrate that staphylococcal food poisoning was caused by a filterable toxin, later called as 'enterotoxin' (Baird-Parker 1990).

2.3.2. Ecology

Staphylococcus constituted a normal part of the micro flora of the animal body, being found on the skin surface, hair, in the nose and throat. Many species have become adapted to life on certain animals and have developed specific biochemical and physiological characteristics to aid their survival. Among the species, *S.aureus* is extremely widely spread, being found on most marine and terrestrial mammals. On the basis of their physiology, resistance to antimicrobial substances and phages, it is possible to separate them into subspecies or ecovars (Baird-Parker, 1990).

2.3.3. Morphology and characteristics

Staphylococci are spherical cells, 0.5 - 1.5 micrometer in diameter, which can occur as single cells, in pairs or as clusters. They are strongly Gram positive, non-motile and asporogenous, capsules may be present in young cultures but are generally absent in stationary phase cells. They are distinguishable from the members of the genus

Micrococcus by their ability to grow anaerobically and to demonstrate fermentable metabolism in contrast to the strictly aerobic metabolism of the obligatory aerobic *Micrococcus* (Baird-Parker, 1990).

2.3.4. Staphylococcal diseases

Staphylococci are parasites of the body surface of warm blood animals. Their ability to cause disease is incidental. The disease they cause can be divided into two broad type:- (1) acute infection which may be localized like pustular or spread (Pyaemia or Septicemia); (2) acute toxaemias, exfoliative toxin, exudative epidermatitis, toxic shock syndrome toxin and staphylococcal food poisoning (Baird-Parker, 1990; Sneath *et al* 1986).

2.3.4.1. Acute infections

The initiation and progress of the disease are dependent on the resistance of the host and the ability of the organism to overcome these. *S.aureus* produces a very wide range of aggressins and exotoxins that are chromosomal or plasmid mediated and include α, β, γ, δ toxins, leucocidins, staphylokinase, hyaluronidase, nuclease, protein A, coagulase, enterotoxin and various surface associated proteins promoting adhesion of cells to host epithelial cells and tissue components. The ability of *S.aureus* to cause infection is multifactorially mediated (Baird-Parker, 1990).

2.3.4.2. Acute toxemia

The causative agents of acute staphylococcal toxemia are specific protein toxins. The specific toxins include toxic shock syndrome toxin 1 (exotoxin C) causing toxic shock syndrome, exfoliative toxin, causing the scaled skin syndrome and the enterotoxin, causing food poisoning (Baird-Parker, 1990; Sneath *et al* 1986).

2.3.5. Staphylococcal exotoxins

S.aureus produces a variety of exoprotiens that contribute to its ability to colonize and cause diseases in mammalian hosts. Nearly all strains secrete a group of enzymes and cytotoxins which include four hemolysins (α, β, γ & δ), nucleases, proteases, lipases, hyaluronidases and collagenases. Catalase production by *S.aureus* functions to inactivate the toxic hydrogen peroxide and free radicals formed by the myleoperoxidase system within host phagocytic cells after the ingestion of the microorganisms (Kloos and Banner, 1999). The free or bound coagulase sometimes called the clumping factor may act to coat the bacterial cell with fibrin, shielding them from opsonization and phagocytosis. Protein-A counters elimination of the pathogen by host polymorphonuclear cells. Thermo nuclease is a heat stable endo or exo nuclease that is capable of hydrolyzing DNA/RNA in host cells. Lipase initiates the spreading to cutaneous and subcutaneous, through phosphatidylinositolspecific phospholipase-C activity. Haemolysins produced by *S.aureus* influence a number of biological functions. α-haemolysins have a lytic effect on red blood cells from an array of animal species. β-haemolysin is a sphingomyelinase acting on lipocarbohydrate sphingomyelin complex (Sandel and McKillip, 2004). The main function of these proteins may be to convert local host tissue into nutrients required for bacterial growth. Some strains produce one or more additional exoprotiens, which include toxic shock syndrome toxin (TSST-1), the staphylococcal enterotoxins, the exfoliative toxin and leukocidin (Dingis *et al,* 2000).

2.3.6. Staphylococcal enterotoxins

Staphylococcal enterotoxins are a group of exotoxins included under the family of pyrogenic toxin super antigens (Dinges *et al,* 2000). They are proteins produced by the Staphylococci under certain conditions in food and culture media. The ingestion of these substances by humans results in what is called Staphylococcal food poisoning. They are a family of major serological type of heat stable enterotoxins and function both as potent

gastro intestinal toxins as well as super antigens that stimulate non-specific T-cell proliferation. Although these are two separate functions localized on separate domains of the proteins, there is a high correlation between these activities (Balaban and Rasooly, 2000).

Staphylococcal enterotoxins are a group of single chain low molecular weight proteins (26,900 - 29,600 daltons), produced by some species of *Staphylococcus*, primarily *S.aureus*. They are similar in composition but are identified as separate proteins due to their difference in antigenicity (Casman *et al,* 1963). To date, 14 different staphylococcal enterotoxin types have been identified which had been designated as A, B, C, D etc, which share structure and sequence similarities (Le Loir *et al,* 2003).

2.3.7. Structure of Staphylococcal enterotoxins

Most of the enterotoxins are neutral or basic proteins with isoelectric points (pIs) ranging from 7 - 8.6, but they have high degree of micro-hetrogeneity when assessed by isoelectric focusing. This has been attributed to enzymatic deamidation. Based on their sequences, biochemistry and functional aspects, all of the staphylococcal enterotoxin (SE) antigenic variants exist as monomeric proteins.

Structural studies have revealed that the molecular topology of all staphylococcal enterotoxins is similar, indicative of their partial sequence conservation (Swaminathan *et al,* 1992). Spectral techniques and computer -assisted structural predictions revealed that staphylococcal enterotoxins A, B, C, and E contain a low content of α–helix(<10%) compared to β-pleated sheets/ β-turn strucure (60-85%) (Smith *et al,* 1974; Bohach *et al,* 1995). The overall shape of SE molecule is ellipsoid with maximal dimensions of 43 by 38 by 32 A° and is folded into two unequal domains. The secondary structure is a mixture of α–helix and β-sheet components. The smaller domains (domain B) have an O/B fold common to staphylococcal nuclease and several other exotoxins. Domain B contains residues near but not including the N- terminus of the mature protein, in the fold of a

Greek–key-β barrel capped by an α–helix. This binding structure has been associated with binding to carbohydrates or nucleic acids in other proteins. In this case, however neither of those two functions has been observed as part of SE activity. The internal β- barrel region is richly hydrophobic, and the external surface is covered by a number of hydrophilic residues. The characteristic SE disulphide bond is located at the end of domain B, opposing the α–helical cap. The resulting loop structure is flexible, it varies between the SEs, depending on the length of loop. Domain A, the larger of two, contains both the amino and carboxyl termini, as well as β-grasp motif. In SEC, this is composed of a five stranded antiparellel β-sheet well. The amino- terminal residues drape over the edge of β-sheet in a loosely attached structure. The interfaces between the A and B domains are marked by a set of α–helices, which form a long groove in the backside of the molecule and a shallow cavity at the top. The long deep groove is similar to TSST-1, and these two structure in SEC3 are the α 5 groove and α 3 cavity respectively (Dinges *et al,* 2000).

Major characteristics of the Staphylococcal enterotoxins (SE)

SE type	ORF* length (bp)**	Precursor Length (aa)+	Mature SE length (aa)	Molecular mass (kDa)	PI***	References
A	774	257	233	27,100	7.3	Betley and Mekalanos 1985; 1988
B	801	266	239	28,336	8.6	Johns and Khan 1988
C1	801	266	239	27,531	8.6	Bohach and Schlievert, 1987
C2	801	266	239	27,531	7.8	Bohach and Schlievert, 1989
C3	801	266	239	27,563	8.1	Hovde *et al.*, 1990.
D	777	258	228	26,360	7.4	Chang and Bergdoll., 1979, Bayles and Iandolo., 1989
E	774	257	230	26,425	7.0	Couch *et al,* 1988
G	777	258	233	27.043	5.7	Munson *et a l,* 1998
H	726	241	218	218	Nd	Su & Wong, 1995
I	729	242	218	25,210	Nd	Munson *et al,* 1998
J	806	268	245	28,565	8.65	Zhang *et al,* 1998
K	729	242	219	25,539	6.5	Orwin *et al,* 2001
L	723	240	215	24,593	8.66	Fitzgerald *et al,* 2001
M	722	239	217	24,842	6.24	Jarraud *et al,* 2001
N	720	258	227	26,067	6.97	Jarraud *et al,* 2001
O	783	260	232	26,777	6.55	Jarraud *et al,* 2001

* ORF Open Reading Frame, ** base pair, *** Iso electric point, + amino acid

2.3.8. Staphylococcal enterotoxin types

2.3.8.1. Staphylococcal enterotoxin A (SEA)

Staphylococcal enterotoxin A (SEA) is the most common toxin implicated in staphylococccal food poisoning (Holmberg and Blake, 1984). The gene for SEA (entA) is carried by a temperate bacteriophage (Betley and Mekalanos, 1985; Borst and Betley, 1994). Hybridization analysis of DNA from 'ent-A converting phage' suggests that this phage integrated into the bacterial chromosome by circularization and reciprocal crossover and that the entA gene is located near the phage attachment site. The '*sea* gene' is composed of 771 base pairs and encodes an enterotoxin A precursor of 257 amino acid residue (Huang *et al,* 1987). A 24-residue N-terminal hydrophobic leader sequence is apparently processed, yielding the mature form of SEA (M 27,100 dalton). There are three SEA isoforms with three different isoelelectric points suggesting variation in processing or post translation modification. The mature SEA is a monomeric, two-domain protein composed of a 13-barrel and a 13-grasp motif (Schad *et al,* 1995), the same general structure found in other enterotoxins.

2.3.8.2. Staphylococcal enterotoxin B (SEB)

The coding region of the gene for SEB (ent B) contains ~ 900 nucleotides (Johns and Khan, 1988). The SEB precursor protein contains 267 amino acids (M 31,400 dalton) and includes an N-terminal signal peptide of 27 amino acids. The entB gene is chromosomal in clinical isolates of *S.aureus* from food poisoning cases (Shafer and Iandolo, 1978). However in other bacterial strains, the gene is carried by a 750kb plasmid (Shalita *et al,* 1977).

2.3.8.3. Staphylococcal enterotoxin type C (SEC)

SECs are a group of highly conserved proteins with significant immunological cross reactivity (Bergdoll *et al,* 1965). The three antigenically distinct SEC subtypes are SEC1, SEC2, and SEC3. The entC3 gene for SEC3 contains 801 bp and encodes a

precursor protein of 267 aminoacids (Hovde *et al,* 1990), containing a 27-residue signal peptide (Bohach and Schlievert, 1989; Hovde *et al,* 1990). The entC 3 gene is closely related to the gene for staphylococcal enterotoxin type C1 with 98% nucleotide sequence identity (Couch and Betley, 1989). SEC3 differs from enterotoxins C 2 and C1 by four and nine aminoacids respectively. *S.aureus* isolated from different animal species produce a unique host specific SEC (Marr *et al,* 1993), suggesting that toxin heterogeneity is due to selection for modified SEC seqence that facilitate the survival of *S.aureus* isolates in their respective hosts (Balaban and Rasooly,2000).

2.3.8.4. Staphylococcal enterotoxin D (SED)

Staphylococcal enterotoxin D (SED) is the second most common serotype associated with food poisoning (Chang and Bergdoll, 1979). The gene encoding SED is entD, which is located on a 27.6- kilobase penicillinase plasmid designated pIB485 (Bayles and Landolo, 1989). The entD gene contains 258 aminoacids including a 30- aminoacid signal peptide. The 228- aminoacid mature polypeptide shows sequence similarity to other staphylococcal enterotoxins (Balaban and Rasooly, 2000).

2.3.8.5. Staphylococcal enterotoxin type E (SEE)

The gene for SEE (ent-E) encodes a 29kDa protein that is apparently processed to a mature extracellular form with a molecular mass of 26 k Da (Couch *et al,* 1988). DNA sequence identity indicates that SEE, SED and SEA are closely related (Vanden Bussche *et al,* 1993). SEA shares high sequence 81% homology with SEA (Balaban and Rasooly, 2000).

2.3.8.6. Staphylococcal enterotoxin type G (SEG)

The ent G gene encodes a 258-amino acid precursor protein that is cleaved to form a toxin with 233 amino acids. The SEG is most similar to SEA, SEB and SEC (Munson *et al,* 1998).

2.3.8.7. Staphylococcal enterotoxin type H (SEH)

SEH is a recently discovered enterotoxin with a molecular mass of 27,300- dalton (Su and Wong, 1995). The amino-terminal of aminoacid sequence of the enterotoxin is unique and immunodiffusion assays do not detect cross- reactivity between SEH and previously identified enterotoxins.

2.3.8.8. Staphylococcal enterotoxin type I (SEI)

The '*ent I*' gene encodes a precursor protein of 242 amino acids (Munson *et al,* 1998). The signal sequence of pre-SEI is cleaved to form a toxin containing 218 aminoacids. SEI has the lowest homology to other SEs.

2.3.8.9. Staphylococcal enterotoxin type J (SEJ)

Characterization of the enterotoxin D-encoding plasmid revealed the presence of an open reading frame (ORF) that encodes an enterotoxin, which has not been identified previously. It is designated staphylococcal enterotoxin J (SEJ) (Zhang *et al,* 1998). The enterotoxin D and J open reading frames are transcribed in opposite directions and are separated by an 895 nucleotide intergeneric region which contains a perfect inverted repeat with each arm of the repeat, having a length of 21 nucleotides. The predicted 269-aminoacid SEJ protein has substantial sequence similarity to SEA, SEE and SED (64-66%). PCR amplification suggests that the 'ent J' determinant may be present on all SED-encoding plasmids (Balaban and Rasooly, 2000).

2.3.9. Properties of staphylococcal enterotoxins

2.3.9.1. Resistance to proteolytic enzymes

Staphylococcus enterotoxins are highly stable. They resist most proteolytic enzymes such as pepsin or trypsin. This keeps their activity in the digestive tract unaffected after ingestion. They also resist chymotrypsin, papain and rennin (Le Loir *et al,* 2003).

2.3.9.2. Heat resistance

Staphylococcal enterotoxins are highly heat resistant (Le Loir *et al,* 2003). Heat stability of the enterotoxins is one of the most important properties in terms of food safety (Denny *et al,* 1971; Hernandez *et al,* 1993). They are thought to be more resistant to heat in foodstuffs, than in laboratory culture medium (Bergdoll, 1983). Heat stability seems to be dependent on the media the toxin is in, the pH, salt concentration and other environmental factors related to the level of toxin denaturation (Balban and Rasooly, 2000). It is an important property of staphylococcal enterotoxins, which means biological activity of the toxin remains unchanged even after thermal processing of food (Holeckova *et al,* 2002). Studies by Denny *et al,* (1966) showed the loss of biological activity after 11 minutes at 121°C (using kitten assay) and after 8 minute at 121°C (using monkey assay).Varnam and Evans,(1991) reported that staphylococcal enterotoxins are not inactivated by irradiation at dose levels acceptable in food products.

2. 3. 10. Enterotoxin detection methods

Many methods have been developed for the detection of staphylococcal enterotoxins, mainly immunological and biological assays. Immunological assays are more sensitive and specific and are the basis for the detection of serologically identified staphylococcal enterotoxins.

2.3.10.1. Biological assays

Biological assays were developed, involving cats and monkeys in the early forties and fifties (Morissette *et al,* 1990). All the staphylococcal enterotoxins were originally detected by their activity in the monkey-feeding assay (Surgalla *et al,* 1953). Intravenous injection of staphylococcl enterotoxins into cats and kittens (Davison *et al,* 1938; Hammon, 1941) were the other methods used but these methods were subjected to non-specific reactions. Also cats and kittens were not as reliable test animals as monkeys. Scheuber *et al,* (1983) used an assay for detection of staphylococcal enterotoxin B assay,

involving intradermal injection of pure toxins with a coloured dye, to highly sensitisized guinea pigs to give a skin reaction. This assay was reported to detect as little as 10 picogram of staphylococcal enterotoxin B in ten to fifteen minutes. The practical difficulties involved in this assay lead to the development of immunological methods. Immunological assays are more sensitive and specific (Su and Wong, 1997).

2.3.10.2. Immunological assays

2.3.10.2.1. Double gel immunodiffusion

Double gel immunodiffusion, also called the Ouchterlony technique (Ouchterlony, 1949) was the first serological assay used for the detection of staphylococcal enterotoxins. Application of the Ouchterlony technique includes microslide, optimum sensitivity plate (OSP) and single radial immunodiffusion methods.

2.3.10.2.2. Radio Immuno assay (RIA)

Radio immuno assay (Miller *et al,* 1978) was the first sensitive method developed for the detection of staphylococcal enterotoxins, at levels of less than 1 ng/ml. This assay is based on the competition for binding sites on specific antibody molecule between radio labeled standards and unlabelled antigens in samples. The major disadvantages of this method include requirement for purified staphylococcal enterotoxins and the use of radioactive materials and special detection equipment.

2.3.10.2.3. Enzyme Linked Immuno Sorbant Assay (ELISA)

The Enzyme Linked Immuno Sorbant assay was first introduced by Saunders and Burlett, (1977). It is currently the most sensitive methods used for the detection of staphylococcal enterotoxins. The major advantage of ELISA is its sensitivity, which is equivalent to the RIA, but without the health hazard associated with radioactivity. Various types of ELISA have been developed for staphylococcal enterotoxin detection, ie competitive ELISA (Kauffman, 1980; Stiffler-Rosenberg and Fey, 1978), non-competitive ELISA and Absorption – Inhibition ELISA (Morissette *et al,* 1990).

2.3.10.2.4. Reverse Passive Latex Agglutination (RPLA) Assay

Reverse Passive Latex Agglutination (RPLA) assay was developed by Shingaki *et al,* (1981). This is based on the visible agglutination of antibody-coated latex particles in presence of staphylococcal enterotoxins. The advantage of this method over ELISA is that it is simple and convenient to use. RPLA is more sensitive than the immunodiffusion assay and enable the detection of Staphylococcal enterotoxin in foods, at the lowest concentration levels implicated in outbreaks of food poisoning (Bankes and Rose,1989). The assay is completed within 24 hours. The RPLA kit is available commercially, for example, kit from Oxoid limited (Basingstoke, Hampshire, UK). Various immunological methods used for the detection of staphylococcal enterotoxins and the various kits used are given in Table 1and 2.

Table 1. Immunological assays for staphylococcal enterotoxins

Assay	Sensitivity	Assay time (hrs)	Quantitation	Reference
Micro-slide	0.1µg/ml	24-72	Semiquantitative	Casman *et al,* (1969)
OSP	0.5µg/ml	18-30	Semiquantitative	Robins *et al,* (1974)
SRD	0.3µg/ml	24	Semiquantitative	Meyer *et al,* (1980)
RIA	1ng/ml	3-4	Quantitative	Miller *et al,* (1978)
ELISA	1ng/ml	4	Quantitative	Freed *et al,* (1982)

Table 2. Diagnostic kits used for the detection of staphylococcal enterotoxins

Kit	Detection Method	Enterotoxin detected	Sensitivity (ng/ml)	Assay time(hrs)
RIDASCREEN	ELISA	A toE	0.2- 0.75	3
SET-EIA	ELISA	A to D	0.1- 1	20
TECRA	ELISA	A to E	1	4
TRANSIA	ELISA	A to E	0.2	1.5
RPLA	RPLA	A to D	0.5-1	20-24
VIDAS	ELFA	A to E	1	1.5

Kit manufacturers

RIDASCREEN: R- Biopharm GmbH, Darmstadt,Germany.

SET-EIA: Diagnostische Laboratorien, Bern Switzerland.

TECRA: Bioenterprises Pty.Ltd., Roseville, New South Wales, Australia.

TRANSIA: TRANSIA- DIFFCHAMB S.A. Lyon, France.

RPLA: Denka Seiken Co.Ltd., Tokyo, Japan.

VIDAS: Bio Merieux Vitek, Inc., Hazelwood, Missouri.

2.3.10.3. Nucleic acid based methods used for the detection of enterotoxigenic *Staphylococcus*

The development of recombinant DNA technology provided a new tool for the detection of microbial pathogens in foods. The DNA based detection of enterotoxigenic staphylococci in foods have been reported by several investigators (Hatakka *et al,* 2000; Johnson *et al,* 1991; Zschock *et al,* 2000). Advancement in molecular methods led to the usage of techniques like Pulse Field Gel Electrophoresis (PFGE), binary typing etc in the characterization of *S.aureus* from different samples (Zadoks *et al,* 2000), but due to disadvantages like requirement of expensive equipments, time consumption, labor intensity etc, simple techniques like Polymerase Chain Reaction (PCR) based ribotyping, Random Amplification of Polymorphic DNA (RAPD) etc are the current methods of choice.

Even though PCR techniques are highly sensitive and specific and allow the detection of microorganisms in a relatively short period with little sample preparations, the technique cannot distinguish viable cells from non-viable cells. Also positive results only indicate the presence of the organism, it doesn't necessarily indicate the presence of staphylococcal enterotoxins in the samples.

2.3.11. Incidence of staphylococcal food poisoning

Staphylococcal food poisoning resulting from the consumption of food contaminated with staphylococcal enterotoxins is the major cause of food borne diseases world wide (Wieneke *et al,* 1993; Knabel, 1995, Su and Wong, 1997; Dinges *et al,* 2000). The foods that are most often involved in staphylococcal food poisoning differ widely from one country to another. Reports from UK showed that 53% of the staphylococcal food poisoning cases were due to meat products, meat based dishes and especially ham, 22% of the cases were due to poultry and poultry based meals, 8% were due to milk products, 7% to fish and shellfish and 3.5% to eggs (Wieneke *et al,* 1993). Haeghebaert *et al,* (2002) reported the incidence of staphylococcal food poisoning in France resulted

from the consumption of meat, 32%, sausages, 22%, pies, 15%, fish and seafood, 11%, egg and egg products, 11% and poultry, 9.5%. The reports from United States showed that 36% of food poisoning was due to red meat, 12.3% to salads, 11.3% to poultry, 5.1% to pastries, and only 1.4% due to milk products and seafoods. In 17% cases, the food involved was unknown (Genigeorgis, 1989). Staphylococcal food poisoning accounts for an estimated 14% of the total out breaks of food borne illness within United States (Holmberg and Blake, 1984; Mead *et al,* 1999). Staphylococcal food poisonig accounts for an estimated 40% of food borne gastroenteritis in Hungary and 20-25% in Japan (Bergdoll, 1979). Outbreaks resulting from improper manufacturing of canned corned beef have been reported in England, Brazil, Argentina, Malta, Northern Europe and Australia. In Great Britain 75% of the 359 cases of food poisoning reported between 1969- 1990 were associated with meat poultry or their products (Wieneke *et al,* 1993).

Staphylococcal food poisoning is a self-limiting illness, presenting with emesis following a short incubation period. Other symptoms include nausea, abdominal cramps, diarrhoea, headaches, muscular cramping and prostration (Jablonski and Bohach, 2001). Symptoms usually develop within 6 hours after ingestion of contaminated food. Death due to staphylococcal food poisoning is not common, but the fatality ranges from 0.03% for general public to 4.4% for more susceptible population such as children and elderly (Holmberg and Blake, 1984). According to the USFDA, effective doses of staphylococcal enterotoxin may be reached when population of *S.aureus* are greater than 10^5cells/ g of food (Anon, 1992). A minimum level of staphylococcal enterotoxin per gram of contaminated food is sufficient to cause symptoms associated with staphylococcal food poisoning (Jablonski and Bohach, 2001).

2.3.12. Toxin types involved in staphylococcal food poisoning from fish and other foods

Out of the fourteen antigenically distinct staphylococcal enterotoxins currently known, enterotoxins A to D are isolated from outbreaks of food poisoning (Bergdoll,

1989). Variation in toxin types involved in staphylococcal food poisoning is reported from different parts of the world. SEA is the most frequent among toxins causing staphylococcal food poisoning (Casman, 1965; Mossel and Netten, 1990; Jay, 1992; Wieneke *et al,* 1993). The preponderance of enterotoxin C producing strains in milk, milk products, meat and human carriers were reported by (Rao, 1977). Varadaraj and Nambudripad, (1982) reported that majority of *S.aureus* isolated from khoa were enterotoxin B producers. Many authors reported that a strain belonging to human biovar produces SEA or SEB (Bergdoll, 1989; Isigidi *et al,* 1992; Mathieu *et al,* 1991 and Sesques, 1994). Contrary to this, Rosec *et al,* (1997) recorded the predominance of SEC production in *Staphylococcus* isolated from foods in France. In India, Rajalakshmi and Rajyalakshmi, (1982) reported, that majority of *S.aureus* recovered from cases of bacterial food poisoning were enterotoxin C producers, either alone or in combination with others. Adesiyun *et al,* (1992) reported the predominance of SEC producing strains followed by SEB and SEA from Nigerian foods. Fang *et al,* (1999) reported the predominance of SEA producing *S.aureus* in vegetarian food products. Many investigators reported the high frequency of SEC and sometimes SED production by strains isolated from dairy products or belonging to bovine or ovine biovar (De Buyser *et al,* 1987; Kenny *et al,* 1993; Marin *et al,* 1992; Orden *et al,* 1992; Valle *et al,* 1990). Fueyo *et al,* (2001) reported SEC as the most frequent toxin from *S.aureus* isolated from human and food samples. Holeckova *et al,* (2002) reported the predominance of enterotoxin B producing isolates (23.5%) from foods.

The predominance of enterotoxins A producing staphylococci from dried fishery products (Sanjeev *et al,* 1985), frozen fishery products (Sanjeev *et al,* 1986, Sanjeev and Surendran, 1994) and from workers of fish processing factories (Sanjeev *et al,* 1987, 1996) from India were reported.

2.3.13. Incidence and enterotoxigenicity of *S.aureus* in fish and fishery products

Staphylococci and especially coagulase positive staphylococci do not constitute the normal flora of fresh marine fish, but only get contaminated either from handlers or from the surface with which they come in contact (Bryan, 1973; Liston, 1980; Sumner *et al*, 1982; Sanjeev *et al,* 1985a). Various authors reported the incidence of staphylococci especially *S.aureus* in fresh fish and shellfish and various fishery products (Ayulo *et al,* 1994; Antony *et al,* 2004; Jeyasekaran and Ayyappan, 2002; Lakshmanan *et al,* 1984; Iyer *et al,* 1986). Their presence in frozen fish and shellfish (Cann, 1977; Silverman *et al,* 1961; Sanjeev *et al,* 1986; Iyer and Shrivastava, 1988) and dried fish and shellfish (Sanjeev *et al,* 1985; Prasad *et al,* 1994) are well documented. Sumner *et al,* (1982) noted the presence of *S.aureus* above 100/g in headless (32%), peeled (35%) and deveined (67%) cooked shrimps processed in Srilanka. Vanden Broek *et al,* (1984) showed *S.aureus* count in the range of 10^2/g in fish fillets from the retail markets of Netherlands.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Materials

3.1.1. Fish and shellfish

Fish, shellfish and fishery products for microbiological examination were procured from various retail fish markets and fish processing factories in Cochin Corporation area (Ernakulam district), except mussels (from Calicut) and oysters (from Kollam). They included fresh, marine and brackish water fishes, prawns, and clams, processed fishes and prawns, dry fish and prawn and fishery products like fish balls, fish kheema, fish finger, fish sausages and fish cutlets. A total of 257 samples have been analyzed. Details of the samples analyzed are given in the following Tables.

Table 3.1.1.1. List of finfish samples analyzed

Sl. No.	Name of fish	Scientific name	No of samples analyzed
1	Thryssa	*Thryssa* sp.	6
2	Pearl spot	*Etroplus suratensis*	5
3	Oil sardine	*Sardinella longiceps*	8
4	Indian mackeral	*Rastrelliger kanagurta*	8
5	Tilapia	*Oreochromis mossambicus*	5
6	Reef cod	*Epinephelus* sp	5
7	Rohu	*Labeo rohitha*	4
8	Seer fish	*Scomberomorus commerson*	6
9	Chinese pomfret	*Pampus chinensis*	4
Total			51

Table 3.1.1.2. List of shrimp samples analyzed

Sl.No.	Local name	Scientific name	No of samples analyzed
1	White shrimp	*Penaeus indicus*	3
2	Tiger prawn	*Penaeus monodon*	4
3	Poovalan	*Metapenaeus dobsoni*	3
4	Karikadi	*Parapaeneopsis stylifera*	2
5	Kazhanthan	*Metapenaeus affinis*	3
6	Scampi	*Macrobrachium rosenbergii*	3
Total			18

Table. 3.1.1.3. List of fresh shellfish samples analyzed

Sl.No.	Local name of shellfish	Scientific name	No of samples analyzed
1	Sea crab	*Portunus pelagicus*	5
2	Mud crab	*Scylla serrata*	5
3	Squid	*Loligo duvaucelli*	4
4	Cuttle fish	*Sepia pharaonis*	4
5	Green mussle (retail arkets)	*Perna viridis*	8
6	Green mussel (Farm)	*Perna viridis*	10
7	Clam	*Villoritta cyprinoides*	11
8	Shucked clam meat	*Villoritta cyprinoides*	9
9	Oyster	*Crassostrea madrasensis*	9
Total			65

Table. 3.1.1.4 List of dried fishes analyzed

Sl.No.	Local name of fish	Scientific name	No of samples analysed.
1	Pallikora	*Saurida* spp	4
2	Flat Mullan	*Leognathus* spp	7
3	Kuttan	*Otolithus* spp	4
4	Shark	*Scoliodon* spp	5
5	Pallathi	*Etroplus maculatus*	5
6	Mullan	*Secutor* species	4
7	Anchovy	*Stolephorus indicus*	6
8	Sole fish	*Cynoglossus* species	5
Total			40

Table.3.1.1.5. List of dried shrimps analyzed

Sl.No.	Local name	Scientific name	No of samples analyzed
1	Poovalan	*Metapenaeus dobsoni*	8
2	Kazhanthan	*Metapenaeus affinis*	8
3	Karikadi	*Parapaeneopsis stylifera*	8
Total			24

Table 3.1.1.6.List of frozen finfish samples analyzed

Sl.No.	Local name	Scientific name	No of samples analyzed
1	Seer fish	*Scomberomorus commerson*	6
2	Pearl spot	*Etroplus suratensis*	5
3	Sea catla	*Lethrinus frenatus*	5
4	Tuna	*Thunnus* sp	5
5	Pomfret	*Pampus argenteus*	3
Total			24

Table 3.1.1.7 List of frozen shrimp samples analyzed

Sl.No.	Item	Localmarket/processing plant	No of samples analyzed
1	BFSPUD (white)	Local market	3
2	IQF (tiger)	Processing plant	4
3	BFSPUD (white)	,,	5
4	BFSPUD (tiger)	,,	5
Total			17

*BSFPUD: Block Frozen shrimp Peeled and un deveined.

*IQF: Individually quick frozen

Table 3.1.1.8. List of other fishery products analyzed

Sl.No.	Item	No of samples analyzed
1	Fish kheema	6
2	Fish finger	2
3	Fish steaks	2
4	Fish cutlet	5
5	Fish sausage	3
Total		18

3.1.2. Bacteriological media

For microbiological examination of fish, shellfish and fishery products, standard culture media were used. (Oxoid, Difco, Himedia and SRL brands).

For detection and characterization of pathogens like *Salmonella, Vibrio cholerae, Vibrio parahaemolyticus* etc, standard culture media described in the Bacteriological Analytical Manual (USFDA, 2001) were used, either by compounding in the Laboratory or by reconstituting the Oxoid/Difco/SRL/Himedia dehydrated media. Media for *E.coli*, coliforms and faecal streptococci described in the Laboratory Manual for Microbiological Examination of Seafood (Surendran *et al,* 2003) were employed.

Composition of the media, diluents, reagents and indicators used in the study are given below.

Composition of Media/diluents / reagents / indicators

a) Media

1. Alkaline peptone water (APW)

Peptone	10.0g
Sodium chloride	5.0g.
Distilled water (DW)	1 Litre

pH: 9.1± 0.1

Dissolved ingredients in 1 litre DW and adjusted pH to 9.1 ± 0.1. Distributed in 225ml. in 500 ml conical flask, and autoclaved at 121°C for 15 minutes.

2. Antibiotic agar

Peptone	10g.
NaCl	10g
Agar	15g
DW	1litre.

pH: 7.2 ± 0.1. Distributed in 100ml quantities in conical flasks. Sterilized at 15 lbs/15min.

3. Baird-Parker medium (BP)

a) Basal medium

Tryptone	10g
Beef extract	5g
Yeast extract	1g
Sodium pyruvate	12g
Glycine	12g
LiCl. $6H_20$	5g
Agar	15g
DW	1 litre

pH: 7.0 ± 0.2. Distributed 100 ml quantities in flasks. Sterilized at 15 lbs for 15 min.

b) Before pouring in to plates, added the following per 100 ml medium.

i) Sterile egg yolk: 5ml of 50% egg yolk

ii) Sterile potassium tellurite: 1 ml of 1% solution.

Allowed the plates to set, Dry at 56°C in an incubator for 45 min., cooled to RT before use.

4. Bismuth Sulphite Agar (BSA)

Peptone	10.0 g
Beef extract	5.0g
Glucose	5.0g
K_2HPO_4	4.0g
$FeSO_4$	0.3g
Bismuth sulphite indicator	8.0g
Brilliant green	0.025g
Agar	15g.
DW	1 litre

pH: 7.6 ± 0.2. Prepared only 500ml. at a time. Dissolved the required ingredients for 500 ml. media in 500 ml. DW, in a litre flask. Heated gently (water bath) with frequent agitation, till just boiling. Cool to 50°C, mixed well and poured into thick plates. Dried at 56°C for 45 min. for use. Kept the plates in refrigerator for one day before use.

5. Blood Agar

Tryptone	15g
Soytone	5g
NaCl	5g
Agar	15g
Distilled water	1 litre

pH: 7.3 ±0.2. Sterilized at 15lbs for 15min. Added 5 ml defibrinated blood to 100ml cooled media, mixed well and poured in to Petri dishes.

6. Blood Agar (Alternate composition)

Peptone	10g
Beef extract	3.0 g
NaCl	5g
Agar	15g
DW	1000ml

pH 7.0 ±0.1. Heated to dissolve, adjusted the pH. Distributed I 100 ml flasks. Autoclaved at 15 minutes 121°C. Added 5 ml defibrinated blood to 100 ml cooled media, mixed well and poured into petridishes.

7. Brain Heart Infusion Broth (BHI) (Difco-Dehydrated medium)

Calf brains Infusion from 200g	7.7g
Brain heart Infusion from 250 g	9.8g
Proteose peptone	10g
Dextrose	2.0 g
Sodium chloride	5.0g
Disodium phosphate	2.5g
DW	1 litre
pH	7.4±0.1

Dispensed in 10 ml quantities in test tubes.

Sterilization	15 lbs 15 minutes.

8. Brilliant Green Agar (BGA).

Proteose peptone	10.0 g
Yeast extract	3.0g
Lactose	10.0g
Sucrose	10.0g
NaCl	5.0g
Phenol red	0.08g
Brilliant green	0.0125g
Agar	15g
DW	1 litre

pH : 6.9 ± 0.2. Heated to boil to dissolve. Sterilized by autoclaving at 115°C for 20 min.

9. Brilliant Green Bile broth 2% (BGLB 2%).

Peptone	1 g
Lactose	1 g
Bile salt	2 g
Brilliant green	0.00133 g*
DW	100 ml

pH : 7.4 ± 0.1 Dissolved the ingredients except Brilliant green in DW, adjusted pH, added 1.33 ml of 0.1%. Brilliant green solution. Dispensed in 4 ml. quantities in small test tubes (10cm x 12mm dia) put Durham's tubes (inverted), plugged with non-absorbent cotton and sterilized at 10 lbs for 20 min.

10. Buffered Peptone Water (BPW)

Peptone	10.0 g.
Sodium chloride	5.0 g.
Disodium phosphate (Na_2HPO_4)	3.5 g.
Potassium di-hydrogen phosphate (KH_2PO_4)	1.5 g.
DW	1 litre

pH : 7.2 ± 0.2. Dissolved in DW, adjust pH, dispensed in 225 ml in 500 ml flasks and sterilized at 15 lb for 15 min.

11. Decarboxylase broth

Basal medium

Yeast extract	0.3g
Glucose	0.1g
Bromocresol purple (BCP)	0.0016 g.*
DW	100ml.

pH : 6.5 ± 0.1. Weighed ingredients except BCP * for 300ml. medium. Dissolved in DW; adjusted pH to 6.5, Added 4.8 ml. of 0.1% Bromocresol purple. Divided into 3 equal volumes of 100ml. each. Added the following amino acids. (One amino acid to one lot of the basal medium). Dispensed in 5 ml volumes in small test tubes and sterilized at 115°C for 20 minutes.

1. L-Lysine Hydrochloride	0.5g.
2. L-Arginine Hydrochloride	0.5.g
3. L-Ornithine Hydrochloride	0.5g

12. DNAse Test agar

Peptone	1g
Beef extract	0.3 g
NaCl	0.5g
Agar	1.5g
DNA Sodium salt	1g
DW	100ml
PH	7.0±0.1

Dissolved, adjusted the pH and sterilized at 15lbs pressure 15 minutes.

13. EC broth

Tryptone	2 g
Lactose	0.5 g
Bile salt (No.3)	0.15 g
K_2HPO_4	0.4 g
KH_2PO_4	0.15 g
NaCl	0.5 g
DW	100 ml

pH : 6.9 ± 0.1. Dissolved, adjusted pH, dispensed in 5 ml quantities in 10cm x 12mm tubes (with Durham's tube inverted), sterilized at 10 lbs for 20 min.

14. Egg yolk Agar

Peptone	1g
Beef extract	0.3 g
NaCl	0.5g
Agar	1.5g
Egg yolk emulsion (50%)	5ml
DW	100ml.

pH: 7.1±0.1. Sterilized at 15 lbs for 15 minutes.
Melted a flask of 100 ml nutrient agar cooled to 50°C, added 5 ml of sterile egg yolk emulsion (50%), mixed well and poured into plates.

15. EMB agar

Peptone	10g
Lactose	10g
K_2HPO_4	2g
Eosin Y	0.4g
Methylene blue	0.065g
Agar	15g
DW	1 litre

pH : 7.1 ± 0.1.Sterilized at 10 lbs/ 20 min.

16. Hektoen's Enteric Agar (HEA)

Protease peptone	12.0g
Yeast extract	3.0g
Lactose	12.0g
Sucrose	12.0g
Salicin	2.0g
Bile salt No.3	9.0g
NaCl	5.0g
Sodium thiosulphate	5.0g
Ferric Ammonium citrate	1.5g
Acid fuchsin	0.1g
Bromothymol blue	0.065g
Agar	15g
DW	1 litre

pH : 7.5 ± 0.2. Dissolved by gentle heat to about to boil. (No autoclaving). Poured into Petri plates. Dried at 56°C for 45 minutes.

17. H & L glucose O/F medium

(Hugh & Liefson's Oxidation /Fermentation Medium-Glucose).

Peptone	1g
NaCl	0.5g
K_2HPO_4	0.4g
Glucose	1g
Agar	0.3g
DW	100ml

pH: 7.1 ± 0.1 Dissolved, adjusted pH to 7.1 and added 1ml of 0.1% solution of phenol red indicator, Dispensed in 8ml quantities in 15cm x 12mm tubes and sterilized at 10 lbs for 20 min.

18. KF agar (For faecal streptococci)

Peptone	10g
Yeast extract	10g
NaCl	5g
Sodium glycerophosphate	10g
Maltose	20g
Lactose	1g
Sodium azide	0.4g
Bromocresol purple	0.015g
Agar	15g
DW	1 litre

pH : 7.2 ± 0.1. Sterilized at 10 lbs for 20min. in 100ml quantities. Before pouring, cooled to 45°C, added 1ml of 1% TTC solution (Dissolved 1g. Triphenyl tetrazolium chloride in 100 ml. DW, sterilized by steaming in an autoclave for 1 hr.)

19. Kligler Iron Agar (KIA).

Peptone	20.0g
Yeast extract	3.0g
Beef extract	3.0g
NaCl	5.0g
Lactose	10.0g
Glucose	1.0g

Ferric citrate	0.3g
Sodium thiosulphate	0.3g
Phenol red	0.05g
Agar	15g
DW	1 litre

pH: 7.4 ± 0.2. Dissolved, adjusted pH, dispensed in test tubes (15ml quantities), sterilized at 115°C for 20 min. After sterilization, kept in a slanting position, so as to get a butt 2.5 cm. deep.

20. Lactose Broth

Beef extract	3 g.
Peptone	5 g.
Lactose	5 g
Distilled water	1 litre

pH : 6.9 ± 0.1. Dissolved the ingredients in DW, dispensed in 225 ml. quantities in 500 ml. conical flasks, pluged with non-absorbent cotton and sterilized at 10 lb. for 20 minutes.

21. Malonate broth

Yeast extract	1g
Ammonium sulphate	2g
K_2HPO_4	0.6g
KH_2PO_4	0.4g
NaCl	2g
Sodium malonate	3g
Bromothymol blue	2.5ml (1% solution)
DW	1litre

pH: 7.0 ± 0.2. Dissolved in DW, distributed in 5ml quantities in small test tubes, sterilized at 15 lbs.for 15min.

22. Mannitol Salt Agar (MSA)

Beaf extract	1 g
Peptone	10g
Mannitol	10g
NaCl	75g.
Phenol red	0.025g
Agar	15g
DW	1 litre

pH. 7.5 ± 0.2; Sterilized at 15 lbs/15 min in 100 ml. quantities. Before pouring, cooled the media to 45°C, added 5ml. of 50% egg yolk per 100 ml. media, mixed well, poured into plates and allowed to set. The plates were dried at 56C for 40 min before use.

23. Milk Agar

Peptone	1g
Beef extract	0.3 g
NaCl	0.5g
Agar	1.5g
Toned milk	5 ml
DW	100ml.
pH	7.0 ±0.1

To Sterile molten nutrient agar medium cooled to 50°C , 5 ml sterile milk was added, mixed well and poured to set.

24. Modified MacConkey Broth (for MPN)

Peptone	2 g.
Lactose	1 g.
Bile salt (No.3)	0.5 g
NaCl	0.5 g.
Bromocresol purple	0.001 g *
Crystal violet	0.0001 g *
Distilled water	100 ml.

pH : 7.4 ± 0.1. Weighed the ingredients except the two dyes (*) for 200 ml medium; Dissolved in 100 ml DW; adjusted pH, added 2 ml. of 0.1% Bromocresol purple and 0.1 ml of 0.2% Crystal violet. Pipetted 10 ml. each into 5 large test tubes (15cm x 25mm dia) for double strength medium. Diluted the rest of the medium to 100 ml by adding 50 ml DW for single strength medium. Pipette 10 ml each into 15cm x 18mm diameter test tubes. Put one Durham's tube (inverted) into each test tube, plugged with non-absorbent cotton and sterilize at 115°C for 20 min. (10 lbs for 20 min.)

25. MRVP medium

Peptone	0.5g
D-glucose	0.5g
K_2HPO_4	0.5g
DW	100ml

pH : 6.9 ± 0.1. Dispensed in 4ml quantities, sterilized at 10 lbs for 20 min.

26. Normal Saline (NS) (Physiological saline)

NaCl.	8.5 g
DW	1 litre

Dispensed in flasks/tubes in the required quantities Sterilized at 15 lbs /15 min.

27. Nutrient Agar

Peptone	10.0g.
Beef extract	3.0g
NaCl	5.0 g
Agar	15.0 g
DW	1 litre

pH: 7.0 ± 0.1. Heated to dissolve, adjusted pH. Distributed in tubes for slants or in 100ml volumes in conical flasks for plating. Autoclaved at 121°C for 15 minutes.

28. Nutrient Broth

Peptone	10.0g
Beef extract	3.0g.
NaCl	5.0 g
DW	1 litre

pH: 7.0 ± 0.1. Heated to dissolve, adjusted pH. Distributed in 225 ml. quantities in 500 ml. conical flasks. Autoclaved at 121°C for 15 minutes.

29. P1 N0 Medium

Peptone	1g
Distilled water	100ml

pH: 7.0 ± 0.1. Heat to dissolved, adjusted pH, distributed in 3 ml quantities in small test tubes .Autoclaved at 121°C for 15 min.

30. Peptone dilution fluid.

Peptone	1g
Distilled water	1000ml

Final pH : 7.0 ± 0.1. Dispensed in 9 ml quantities in test tubes. Autoclaved 15 min at 121°C.

31. Phenolphthalein phosphate agar

Peptone	1g
Beef extract	0.3 g
NaCl	0.5g
Phenolphthalein di-phosphate	I ml of 1% solution
Agar	1.5g
DW	100ml
pH	7.0 ±0.1

To the dissolved nutrient agar, 1% solution of Phenolphthalein diphosphate sodium salt (BDH) I ml was added and sterilized at 15 lbs 15 minutes

32. Phosphate Buffer diluent

KH_2PO_4	34g
Distilled water	500ml

Dissolved. Adjusted pH to 7.2 ± 0.1 with 1 N NaOH, diluted volume to 1 litre with distilled water (stock solution). Took 1.25 ml of the above stock solution and diluted volume to 1 litre with distilled water. Dispensed in flasks or tubes and sterilize at 15 lbs for 15min.

33. Purple Broth Base (For Sugar Fermentation).

Peptone	10g
NaCl	5g
Bromocresol purple	0.02 g
DW	1 litre

Adjusted pH to 7.0 ± 0.2. Add the required sugar to 1% level, dissolved and distributed in small test tubes containing inverted Durhams tubes. Sterilize at 115°C for 20 minutes

34. Selenite cystine broth.

Tryptone	5.0g
Lactose	4.0g
$Na_2 HPO_4$	10.0g
L-Cystine	0.01g
Sodium biselenite	4g.
DW	1 litre

pH: 7.1 ± 0.1. Dissolved sodium biselenite in 1 litre DW and add the other ingredients. Warm to dissolve, dispensed in test tubes in 10ml. quantities. Sterilized by steaming in an autoclave for 15 min.

35. Simmon's citrate agar

Sodium citrate	0.2 g
NaCl	0.5g
K_2HPO_4	0.1g
$NH_4H_2PO_4$	0.1g
$MgSO_4$	0.02g
Bromothymol blue	0.008g
Agar	1.5g
DW	100 ml

pH: 7.0 ± 0.2. Dissolved, adjusted pH, dispensed in 4ml quantities in small test tubes, sterilized at 15lbs/15 min. Allowed to set in a slanting position overnight.

36. Sugar fermentation media.

Peptone	10.0 g.
Sodium chloride	5.0 g
DW	1 litre

pH: 7.2 ± 0.1. Dissolved ingredients in 1 litre of distilled water. Added 10ml of Andrade's Indicator. Adjusted pH. Added the required sugar to 1% level, dissolved and distributed in small test tubes containing inverted Durhams tubes. Sterilized at 115°C for 20 minutes.

37. Tergitol-7 agar (T-7 agar).

Peptone	10g
Yeast extract	6 g
Beef extract	5 g
Lactose	20g
Tergitol-7	0.1g
Bromothymol blue	0.05g
Agar	15g
DW	1 litre

pH : 7.2 ± 0.2. Sterilized at 10lbs for 20 min in 100ml quantities. Before pouring the plates, after melting, add 0.25ml of 1% solution of sterile Triphenyl Tetrazolium Chloride (TTC) per 100 ml media.

38. Tetrathionate broth

a) Base

Beef extract	0.9 g
Peptone	4.5g
Yeast extract	1.8g
NaCl	4.5g
$CaCO_3$	25.0g
Sodium thiosulphate	40.7g
DW	1 litre

pH: 8.4 ± 0.2.Dissolved the ingredients in DW, heated to boil, cooled below 45°C and added 20ml of iodine solution (see below). Mixed and tubed in 10 ml quantities. No autoclaving required.

Note: The prepared base will keep for several weeks at 4°C; but should be used soon after addition if iodine.

b) Iodine solution

Iodine crystals	6g.
Potassium Iodide (KI)	5g.
DW	20 ml

Ground iodine and KI in a mortar and added water to dissolve.Added the iodine solution to the basal media.

39. Thiosulfate Citrate Bile Salt Sucrose Agar (TCBS)

Yeast extract	5.0g.
Peptone	10.0g
Sucrose	20.0g.
Sodium thiosulphate	10.0g.
Sodium Citrate Dihydrate	10.0g.
Sodium cholate	3.0g.
Ox-gall	5.0g
Sodium chloride	10.0g.
Ferric citrate	1.0g
Bromothymol blue (BTB)	0.04g.*
Thymol blue (TB)	0.04g.*
Agar	15.0g.
DW	976ml.

pH: 8.6 ± 0.1. Weighed all ingredients except the dyes (*), added DW. Heated to boiling with agitation to obtain complete solution. Added 20ml 0.2% BTB and 4 ml 1% TB. Heated to boil again. Did not autoclave; Cooled to 45°C. Adjusted pH to 8.6 and poured 15-20ml into petridishes; allowed to set and dried at 56°C for 45 min.

40. $T_1 N_0$ and $T_1 N_3$ medium.

Trypticase	2.0 g
DW	200 ml.

pH: 7.2 0 ± 0.2. Dissolved; adjusted the pH ; divided into 2 lots. To 1 lot added 3 g. NaCl , dissolved and dispensed in 5 ml quantities in test tubes . This is T1N3 medium. The 2nd lot was also tubed in 5 ml. quantities in test tubes. This is T1N0 medium. Sterilized at 121°C for 15 min.

41. Trypticase salt broth (With various NaCl level)

Trypticase	10 g
DW	1L.

Dissolved, Adjusted pH at 7.2 ± 0.2. Divided into 5 lots of 200ml each. Added 2,6,12,16,20 g NaCl each to each set respectively to make media with salt concentration of 3, 6, 8 &10 %. Distributed 5 ml. quantities in test tubes. Sterilized at 121 C for 15 min.

42. Triple Sugar Iron Agar (TSI)

Peptone	20.0g.
Yeast extract	3.0g.
Beef extract	3.0g.
Lactose	5.0g.
Sucrose	10.0g.
Glucose	1.0g.
Ferric citrate	0.3g.
Sodium thiosulphate	0.3g.
Phenol red (0.2% soln:)	12 ml.*
Agar	12.0g.
DW	988 ml.

pH: 7.4 ± 0.2. Added all ingredients except phenol red to distilled water, mixed and heated to boiling to dissolve. Cool to 50-60°C and adjust pH to 7.4 ± 0.2. Added phenol red. Dispensed in test tubes one-third full and plugged. Autoclaved at 115°C for 20 min. Cooled tubes in slanted position overnight to obtain a butt 2.5cm deep.

43. Tributyrin Agar

Peptone	0.5g
Yeast extract	0.3g
Agar	1.5g
DW	100ml
Tributyrin	2 ml
PH	7.5±0.1

Dissolved the ingredients except tributyrin. Adjusted the pH. Added 2 ml tributyrin. Blended in a waring blender to emulsify. Sterilized at 15 lbs for 15 minutes

44. Tryptone broth (Indole medium)

Tryptone	1 g
NaCl	0.5 g
DW	100 ml

pH: 7.1 ± 0.1. Distributed 5ml quantities in 10x12 mm tubes. Sterilized at 15 lbs for 15 min.

45. Tryptone Glucose Agar (TGA)

Tryptone	0.5 g
Beef Extract	0.3 g
NaCl	0.5 g
D-Glucose	0.1 g
Agar agar	1.5 g
Distilled water	100 ml

pH: 7.1± 0.1.Sterilized at 121°C for 15 min.

46. Urea Agar.

Peptone	1g
Dextrose	1g
Sodium Chloride	5g
Pot.dihydrogen phosphate	2g
Phenol red	0.012g
Urea	20g
Agar Agar	15g
Distilled water	1000ml.

pH : 6.8 ± 0.1. Dissolved the ingredients, except urea and phenol red, in distilled water by boiling, adjusted the pH, added 6.0 ml. of 0.2% solution of phenol red, distributed in 9.5 ml. quantities in test tubes, sterilized at 115°C for 20 min. Cooled to 50°C and aseptically added 0.5 ml. of a 40 % solution of urea per tube mixed well and allowed to cool to form slants.
(Urea solution is prepared by dissolving 20g Urea in Sterile Distilled water and filter sterilized. Phenol red solution is prepared in 50 % ethyl alcohol).

47. Violet Red Bile Glucose Agar (VRBGA).

Yeast extract	3g
Peptone	7g
NaCl	5g
Bile salt (No.3)	1.5g

Dextrose (D-glucose)	10g.
Neutral red	0.03g
Crystal violet	0.002g
Agar	15g.
DW	1 litre

pH : 7.4 ± 0.1. Sterilized at 10 lbs for 20 min.

48. Wagatsuma Agar

Peptone	10g
Yeast Extract	3g
NaCl	70g
K_2HPO_4	5g
Mannitol	10g
Crystal violet	0.001g
Agar	15g
Distilled water	1litre

pH: 8.0 ± 0.2. Heated with agitation to dissolve agar, adjusted pH, steamed for 30min. (Not autoclaved). Added 5% by volume red blood cells to the cooled medium. Mixed and poured into petridishes. Dried the plates before use.

49. Xylose-Lysine Desoxycholate Medium (XLD).

Yeast extract	3.0g
L-Lysine Hydrochloride	5.0g
Xylose	3.75g
Lactose	7.5g
Sodium desoxycholate	1.0g
NaCl	5.0g
Sodium thiosulphate	6.8g
Ferric Ammonium Citrate	0.8g
Phenol red	0.08g
Agar	15g
DW	1Litre

pH: 7.4 ± 0.2. Heated with agitation till boiling, no over heating, cooled to 50°C, poured into plates. Dried at room temperature for 2 h. (Used on the day of preparation).

Staining Solutions

1. Crystal violet for Gram's stain

Solution A

Crystal violet	2g
Ethyl alcohol	20ml
Dissolved	

Solution B

Ammonium oxalate	0.8g
DW	80ml
Dissolved	

Mixed solutions A&B, filtered; kept overnight before use.

2. Gram's iodine (For Gram's staining)

KI	2g
Iodine crystals	1g
DW	300ml

Ground Iodine and KI together in a glass mortar; Dissolved and filtered.

3. Safranine (for Gram's stain)

Safranine	1g
Ethyl alcohol	40ml
DW	360ml

Dissolved safranine in alcohol, added DW and filtered.

Indicator Solutions

1. Andrade's indicator (for Sugar Fermentation Tests)

Acid fuchsin	0.5g
DW	100ml
1N.NaOH solution	16ml.

Dissolved acid fuchsin in DW, added the NaOH solution. Kept overnight.

2. Bromocresol Purple Indicator (For Purple Broth base)

Bromocresol purple	0.2g

Alcohol	10ml
Distilled water	90ml

3. Bromocresol Purple Solution (For MacConkey broth)

Bromocresol Purple	100mg
Alcohol	100ml

4. Bromothymol blue indicator. (for pH adjustment)

NaOH	40mg
Bromothymol blue	250 mg
DW	100ml

Dissolved 40 mg NaOH in 100ml DW; dissolved the bromothymol blue. Adjusted the colour to bottle green (pH-7) by 1N.HCl. filtered.

5. Brilliant Green Solution(For BGBLB)

Brilliant Green	100mg
Distilled water	100ml

6. Crystal Violet Solution (For Mac Conkey Broth)

Crystal Violet	1g
Distilled water	100ml

7. Methyl Red indicator (for MR test)

Methyl red	50mg
Alcohol	150ml
DW	100ml

Dissolved Methyl red in alcohol, diluted with DW and filtered if necessary.

Test: To a tube of 48hr. (or more) old bacterial culture in MRVP medium, added 5 drops of Methyl red indicator. A red colour is +ve test; yellow is negative.

8. Potassium tellurite Solution (For BP Agar)

Potassium Tellurite	1g
Sterile distilled water	100ml

Steam for 30min.in a water bath

Test Reagents and Tests

1. Kovac's cytochrome oxidase reagent

N:N:N:N.Tetramethyl-p-phenylene diamine-hydrochloride	100mg.
DW	10 ml

Dissolved. Impregnated filter paper with the reagent and drained. Kept the test paper in a refrigerator away from light.

Test: Smeared a little of the young bacterial culture on the test paper. Development of a blue colour within a few seconds indicated a positive test for cytochrome oxidase (Oxidase).

2. Kovac's Indole Reagent

p-Dimethyl amino benzaldehyde	5g
N-butyl alcohol(or amyl alcohol)	75ml.

Dissolved. Add 25ml. concentrated HCl. Filtered if necessary. Kept in amber coloured bottle overnight before use.

Test : To a tube of 48 hr old Tryptone broth culture added 0.2 ml of the reagent. Shook and allowed to stand. Development of a pink or red layer at the top indicated a positive test for Indole.

3. VP test reagents and test

Solution A

α-naphthol	0.25g
Alcohol	5ml.

Solution B

KOH	2g
DW	5 ml.

Test: In a small test tube took 1 ml of 48 hr. old bacterial culture grown in MRVP medium added 0.6ml solution A and 0.2ml solution B. Shook well, Put a small crystal of creatine. Shook and allowed to stand up to 4 hrs. Eosine pink colour indicated a +ve VP test.

Preparation of sterile egg yolk (50%)

Took 5 hen's egg, washed free of dirt with vim and water, wiped dry and kept immersed in alcohol (rectified spirit) in a 1 litre beaker for 2 hours. Drained off the

alcohol into a bottle. Took out the egg (one at a time), made a small opening at one end using a sterile scalpel, and poured out all the egg white carefully. Carefully broke the shell a little more and transfered the egg yolk (yellow) into a sterile conical flask. Each egg will provide about 15 ml. of egg yolk. Added an equal volume of sterile Normal saline, agitated well and allowed to stand. Pipetted 5 ml. each of the egg yolk saline into sterile test tubes plugged with sterile cotton and kept in a refrigerator (at 5-8°C).

3.1.3 Toxin Assay kits

For detection and assay of *Staphylococcus* enterotoxins SET-RPLA kits (Oxoid,UK) were employed.

3.1.4 PCR Reagents and primers

PCR Buffer	(Biogene, USA)
dNTP's	(Finzyme, Finland)
Taq polymerase	(Biogene, USA)
DNA ladder	(Fermentas , USA)
Primer	(Fermentas, USA)

3.1.4.1. Agarose gel electrophoresis reagents

1. Agarose (Molecular grade, Sigma Aldrich, USA)
2. Tris-Acetate buffer (Tris base 242g (Sigma, USA), glacial acetic acid 57.1ml (SRL, India), ethylene diamine tetra acetate (EDTA, SRL, India) 100ml (0.5M, pH 8).
3. Ethidium bromide (10mg/ml)
4. 6X gel loading buffer (Genei. Bangalore)
5. Molecular marker (100bp, Gene Ruler, Fermentas International, Germany).
6. TE buffer (Tris 10mM, EDTA 1mM, pH 8)

3.1.5. Antibiotic test discs (Oxoid, Himedia, India)

Chloramphenicol(30mcg), Penicillin(10 IU), Erythromycin(15mcg), Methicillin (5mcg), Novobiocin(30mcg), Tetracycline (30mcg), Oxytetracycline (30mcg), Neomycin (30mcg), Nalidixic acid(30mcg), Oxolinic acid(2mcg), Vancomycin(10mcg), Sulphamethazole(25mcg).

3.1.6. List of type cultures from National Collection of Industrial Microorganisms, Pune

1. NCIM-2079 *S.aureus*
2. NCIM-2720 *S.aureus*
3. NCIM-2121 *S.aureus*
4. NCIM- 2127 *S.aureus*
5. NCIM- 2122 *S.aureus*

3.1.7. Other chemicals and Biochemicals

All other chemicals and biochemical's including enzymes were Sigma Aldrich/ E Merck/ Himedia brands.

3.1.8 Equipments

In addition to the standard equipments of a microbiological laboratory, the following special equipments from the biotechnology laboratory of CIFT were also used.

1. Refrigerated centrifuge 5804R Eppendorf (Germany)
2. Spectrophotometer (Varian India Pvt Ltd)
3. Thermal cycler (Eppendorf, Germany)
4. Microscope (Labomed)
5. Alpha imager (Alpha Innotech Corporation ,USA)
6. Water activity meter (Lufft, Germany)

3.2 Methods

The fish and shellfish samples were analyzed for microbiological parameters quantitatively as well as qualitatively as per standard methods (USFDA 2001, Surendran *et al,* 2003).

3.2.1 Quantitative analysis

The quantitative analysis was carried for Total Plate Count (TPC), Total Enterobacteriaceae count (TEC), *E.coli*, *Faecal Streptococci* count and *Staphylococcus aureus*. MPN analysis was done for total coliforms and faecal coliforms.

3.2.1.1. Preparation of Sample

From each fish and shellfish sample, 10g muscle was aseptically cut and transferred into a sterile polythene stomacher bag and blended with 90ml sterile normal saline in a stomacher homogeniser (Stomacher 400, Seaward medicals, UK.) at 230 rpm for 60 seconds. This was the 10^{-1} dilution of sample. Using a sterile 1 ml pipette, one ml of the homogenate was aseptically transferred into 9 ml normal saline in test tube. The tube was mixed well using a vortex mixer (10^{-2} dilution). Similarly, further dilutions required for inoculation was prepared by this decimal serial dilution process.

3.2.1.2. Total Plate Count (TPC) Pour plate method

One ml each of the appropriate dilution (3.2.1.1) was pipetted to marked sterile petridishes in duplicates for each dilution. About 15-18 ml of molten and cooled (40-45°C) Tryptone Glucose Agar (TGA) was poured to each plate. The contents were mixed well by slow rotation of the petridishes and allowed to set. The set plates were then incubated at 36±1°C for 48 hours in an inverted position in an incubator. After the incubation period, the individual bacterial colonies on each plate were counted using a Quebec colony counter. The average counts of the duplicates were calculated. The total plate count (TPC/gram) of the sample was calculated using the relation,

TPC/g of sample = Average count X Dilution factor.

In the case of crowded plates or plates with fewer colonies, the TPC was calculated as described in Bacteriological Analytical Manual (USFDA, 2001).

3.2.1.3. Total Plate Count (TPC) by Spread plate method

TGA was molten, cooled and poured in to sterile petridishes allowed to set and dried at 56°C for 45 minutes, then cooled to room temperature (28±2°C) to obtain pre-set TGA plates. Appropriate dilutions were pipetted (0.5 ml each) into preset TGA plates in duplicates for each dilution. The inoculam was spread well over the surface using a sterile bent glass rod. The plates were incubated at 36±1°C for 48 hours in an inverted position.

After incubation the individual bacterial colonies were counted using a Quebec colony counter. The average count of the duplicates was calculated. The total plate count (TPC/g) of the duplicates was calculated using the relation,

TPC/g of the sample = Average count X 2 X dilution factor

3.2.1.4. Total Enterobacteriaceae Count (TEC)

One ml each of appropriate dilutions prepared as per 3.2.1.1 was pipetted into sterile petridishes taken in duplicates for each dilution. About 15-18 ml of molten and cooled (40-45°C) Violet Red Bile Glucose agar (VRBGA) was poured to each plate. The inoculam was mixed well with the medium and allowed to set. The set plates were incubated at 36±1°C for 18-24 hours in an inverted position in an incubator. After the incubation period, the red, small (2-4 mm dia) bacterial colonies were counted as Enterobacteriaceae colonies. The average count of the duplicate plates was calculated. The total Enterobacteriaceae count of the samples was calculated using the relation

TEC = Average count X Dilution factor.

3.2.1.5. Faecal streptococci count

One ml each of the appropriate dilutions (ref: 3.2.1.1) was pipetted to sterile petridishes taken in duplicates for each dilution. About 15-18 ml molten and cooled (40-45°C) Kenner Faecal Streptococci Agar (KF) was poured to each plate. The medium was mixed well with inoculam and allowed to set at room temperature. The set plates were then incubated at 36±1°C for 48 hours in an inverted position. All the surface and subsurface red to pink, small colonies (1-2 mm) were counted as faecal streptococci. The average count of the duplicates was calculated. The *Faecal streptococci* count/g of the sample was calculated using the relation,

Faecal streptococci count/g of the sample = average count X dilution factor

3.2.1.6. *Staphylococcus aureus* count

Appropriate dilutions (0.5 ml) of each dilution of fish homogenate (3.2.1.1) were pipetted into pre set and dried Baird- Parker (BP) agar plates in duplicates for each dilution. The inoculam was spread well over the surface using a sterile bent glass rod. The plates were incubated at 36±1^{oo}C for 36-48 hours. All the jet-black colonies with a white margin and zone of clearance were counted as *S.aureus*. The average count of the duplicates was calculated. The *Staphylococcus aureus* count was calculated using the relation

Staphylococcus aureus count/g of the sample = average count X 2 X dilution factor

3.2.1.7. *Escherichia coli (E.coli)* count

Appropriate dilutions (0.5 ml) from the sample homogenate (3.2.1.1) were pipetted into pre-dried plates of Tergitol –7 agar (T-7) in duplicates for each dilution. The inoculam was spread well over the surface using bent glass rod. The plates were then incubated at 36±1°C for 18-24 hours. Lemon yellow occasionally with rust brown center, circular, non- mucoid colonies were counted as *E.coli*. These colonies were further confirmed by streaking on EMB plates and by IMVIC test.

E.coli count /g of the sample = Average count X 2 X dilution factor.

3.2.2. Most probable number methods for coliforms and *E.coli*

When the expected number of Enterobacteriaceae including *E.coli* and faecal coliforms were less than 100/g in the sample, MPN methods were used to detect them. The three tube, three step MPN method was employed (Surendran *et al,* 2003).

Step 1: For presumptive total coliforms

From the 10^{-1} dilution of the sample homogenate (90ml saline + 10g sample, prepared as in 3.2.1.1), 10 ml each of the inoculam was transferred to each of the 3 tubes of double strength Mc Conkey broth. One ml each of the sample was pipetted to three 10 ml single strength Mc Conkey broth tubes and one ml of 10^{-2} dilution to three 10 ml

single strength Mc Conkey broth. The tubes were incubated at 36±1°C for 24 hours. Tubes producing acid and gas from lactose broth (gas in the Durham's tube) were taken as positive. Positive tubes were noted, and compared with standard MPN table to get MPN values for presumptive total coliforms count.

Step II: For confirmed coliforms

A loopful of culture from the positive tubes of step I were inoculated to Brilliant Green Lactose Broth (BGLB broth 2%) and incubated at 36±1°C in a serological water bath for 24 hours. The number of tubes showing growth and gas production in Durham's Tubes was noted. They were taken as positive. The results were compared with standard MPN table to obtain the MPN value for confirmed coliforms.

Step III: ***Faecal coliforms and E.coli***

From the positive tubes of step II, one loopful each was inoculated to EC broth and Tryptone broth and incubated at 44.5±0.5°C for 24 hours in serological water bath. Turbidity and gas production in EC tube indicated positive for faecal coliforms. Results were compared with standard MPN table to get the MPN value for faecal coliforms.

Into the tryptone broth tube , one ml of Kovac's Indole reagent was added and shaken and allowed to stand. A deep red ring at the top indicated indole positive reaction. *E.coli* will produce indole at 44.5±0.5°C in 24 hours. The results were compared with standard MPN table and results were recorded as MPN *E.coli*.

3.2.3. Detection and Identification of *Salmonella*

For detection of *Salmonella* the following procedures were employed (USFDA, 2001).

1. Pre-enrichment

Twenty five gram of the fish /shellfish sample was transferred into a sterile stomacher bag, added 225 ml lactose broth and blended for 30 seconds in a stomacher blender.

The contents were transferred aseptically into a sterile 500 ml conical flask and incubated at 36 ±1°C for 18-24 hours.

2. Selective enrichment

Pipetted 1 ml from the above pre-enriched culture to 10 ml each of selective enrichment medium viz: Selenite cysteine broth and Tetrathionate broth. Incubated at 36±1°C for 18-24 hours.

3. Selective plating

Streaked one loopful each from the selective enrichment culture (above) on to pre-dried selective plating medium, viz. (1) Brilliant Green Agar(BGA), (2) Bismuth Sulphite Agar (BSA), (3) Hekton's Enteric Agar (HEA), (4) Xylose Lysine Desoxycholate Agar (XLD). Incubated at 36±1°C for 24-48 hours. Examined the plates for typical Salmonella colonies as follows:

a) BGA: Smooth, low, convex, moist pink colonies; surrounding medium bright red.

b) BSA: Brown, grey to black colonies with metallic sheen, surrounding medium brown to black.

c) HEA: Colonies blue or green with or with out black center.

d) XLD A: Colonies red (pink) with or without black centers.

4. Inoculation to Urea, TSI and LIA Agar

Selected 2 to 3 typical or suspected colonies from each selective agar plates above. Lightly touched the center of the colony to be picked with sterile needle and inoculated in Triple Sugar Iron agar (TSI agar) and Lysine Iron Agar(LIA) by streaking the slant and stabbing the butt twice. Incubated the TSI slants at 36±1°C for 24 hours and LIA slants at 36±1°C for 48 hours. Urea Agar slants were inoculated by streaking the slant and incubated at 36±1°C for 24 hours.

Retained all the cultures giving positive reactions for further test and confirmation by the following biochemical tests.

1. Lysine decarboxylation test
2. Sugar fermentation:-

   Dulcitol, Glucose, Lactose, Sucrose, Salicin
3. Malonate utilization test
4. Indole test
5. MRVP test
6. Simmon's citrate utilization test
7. Gram reaction
8. Motility test.

5. Serological confirmation

All cultures giving typical biochemical reactions were confirmed by agglutination test with *Salmonella* polyvalent somatic (O) antiserum as follows: -

Marked two sections about 1X2 cm each, on a clean oil free glass slide, emulsified a loopful of the above culture with 2 ml of 0.85% saline in a test tube. Added one drop of the culture suspension to both the marked sections on the slide. Added one drop of saline (0.85% NaCl in DW) to one section and one drop of the Salmonella polyvalent O antiserum (Difco,USA) to the other section. Using a loop, mixed culture suspension with saline solution in one section and with the antiserum on the other section. Tilted the mixture in back and forth motion for one minute and observed against dark background in good illumination for agglutination. Any degree of agglutination was considered a positive reaction. *Salmonella* was identified based on the reaction given in Table 3.2.3.

Table. 3.2.3: Reactions of *Salmonella*

Test	Result
Gram stain	Gram –ve short rod
Motilirty	Motile
TSI	Alkaline slant (K) Acid butt (A), H_2S positive
LIA	Alkaline slant, alkaline butt, H_2S positive
Urease	Negative
Indole	Positive
Glucose	Acid and Gas
Lactose	Negative
Sucrose	Negative
Dulcitol	Acid and gas
Salicin	Negative
MR test	Positive(Red)
VP test	Negative
Lysine decarboxylase	Positive(Purple colour)
Malonate utilization	Negative
Citrate	Positive (Blue)
Salmonella polyvalent(O) antisera	Agglutination positive

3.2.4. Detection and identification of *Vibrio cholerae*

For detection of *Vibrio cholerae* the following procedure, (USFDA, 2001) was adopted.

1. Enrichment

25g of the sample was blended with 225 ml of Alkaline Peptone Water (APW) in a stomacher blender and contents transferred aseptically to a sterile 500ml conical flask and incubated at 36±1°C for 24 hours.

2. Selective plating

After 6-8 hours and 16-24 hours of incubation, without shaking the flask, loopfuls from the surface growth was withdrawn and streaked on to pre-set Thio-sulphate Citrate Bile salt Sucrose (TCBS) agar. Incubated the plates at 36±1°C for 18- 24 hours. Examined the plates for typical *Vibrio cholerae* colonies. viz; Large (2-3 mm dia), smooth, yellow, slightly flattened with opaque center and translucent peripheries.

3. Isolation of colonies

Picked typical colonies to nutrient agar slants and incubated at 36±1°C for 18- 24 hours.

4. Inoculation to TSI and KIA

Inoculated the isolated cultures into TSI and KIA by stabbing the butt and streaking the slant. Incubated at 36±1°C for 18- 24 hours. Observed for typical reactions of *Vibrio cholerae (See Table 3.2.4).*

5. Biochemical tests

For biochemical confirmation of the isolated cultures the following test were done.

a) Salt tolerance (Tryptone broth with 0% and 3% NaCl)

Inoculated into T1N0 and T1 N3 media and incubate at 36±1°C for 18-24 hours.

b) Hugh & Leifson Oxidative/Fermentative test (H&L- O/F) test: for utilization of glucose.

c) Cytochrome oxidase test

d) Fermentation of carbohydrates

Glucose, Sucrose, Mannitol, Inositol, Arabinose

e) Decarboxylase tests

Inoculated a loopful of the cultures to the following three amino acid media added sterile liquid paraffin (~1 cm height) and incubated at 36±1°C up to 4 days. Examined for colour change every day.

a. Lysine broth

b. Arginine broth

c. Ornithine broth

f) Sensitivity to Vibriostatic agent (O/129 compound)

g) Serological confirmation: (Agglutination with polyvalent *V.choleraee* O antiserum).

Vibrio cholerae was confirmed based on the reaction given in Table 3.2.4.

Table.3.2.4. Characteristic reactions of *Vibrio cholerae*

Test	Reaction
TSI	Acid slant& Acid butt
KIA	Alkaline slant & Acid butt
T1No & T1N3	Growth positive in both
H&L Glucose O/F test	Fermentative (Acid only, no gas)
Cytochrome oxidase	Positive
Gram stain	Gram negative short rods
Motility	Motile
Indole	Positive
D-Mannose	Acid only
D-Mannitol	Acid only
L-Inositol	Negative
Glucose	Acid only
Sucrose	Acid only
Arabinose	Negative
L-lysine decarboxylase	Positive (Purple colour)
L-Arginine dihydrolase	Negative (Yellow colour)
L-Ornithine decarboxylase	Positive (Purple colour)
Sensitivity to O/129 (10μg) disc	Positive (Zone of clearence)
Sensitivity to O/129 (150 μg) disc	Positive (Zone of clearence)
H_2S production (Black colour in TSI)	Negative
Agglutination with polyvalent *V. cholerae* O antisera	Positive for O1 *V.cholerae*
	Negative for non-O1 *V.cholerae*

3.2.5. Detection and identification of *Vibrio parahaemolyticus*

For the detection of *Vibrio parahaemolyticus* the following procedure was adopted (USFDA, 2001).

1. Enrichment

25g of the sample was homogenized with 225 ml of alkaline peptone water containing 3%salt in a stomacher blender and transferred to a sterile 500ml flask and incubated at 36±1°C for 24 hours.

2. Selective plating

Streaked loopful of the APW culture (above) on pre-set plates of TCBS agar (with 3% salt) and incubated at 36±1°C for 24 hours. Examined the colonies on TCBS agar. Viz; Round, 3-5 mm green or blue green colonies with blue or green center.

3. Isolation of colonies:

Picked typical colonies on to a nutrient agar slants with (3% salt) and incubated at 36±1°C for 24 hours. The following biochemical tests were carried out with these pure cultures.

4. Bio chemical and Growth studies tests

(i) TSI agar with 3% salt

(ii) KI Agar with 3% salt

(iii) Gram staining

(iv) Cytochrome oxidase test

(v) H& L Glucose medium(O/F) test

(vi) Halophilicity test in. 0%,3%,6%, 8%,10% NaCl

(vii) MRVP test

(viii) Sugar fermentation test; Glucose, Sucrose, Mannitol, Arabinose, Inositol

(ix) Gelatin liquifaction test

(x) Sensitivity to Vibrio static agent (O/129) discs

(xi) Kanagawa reaction for pathogenicity was done as follows

Prepared plates of Wagatsuma agar (NaCl 7%), spot or stab inoculated from overnight broth culture. Incubated at 36±1°C for 18-24 hour. Checked for haemolysis. A clear zone indicated a positive reaction (β-haemolysis). A discolouration (α – haemolysis), and no change (γ – haemolysis) were considered negative.

xii). Sensitivity to Vibrio static agent (O/129) discs (Oxoid ;U.K.)

xiii) Growth at 42°C.

Vibrio parahaemolyticus was confirmed based on the reaction given in Table 3.2.5.

Table.3.2.5. Characteristic reactions of *Vibrio parahaemolyticus*

TSI Agar	Alkaline slant and acid butt, No H_2S
KI Agar	Alkaline slant, acid butt , No H_2S
Gram reaction	Gram negative short or curved rods
Motility	Motile
Cytochrome oxidase	Positive
Catalase	Positive
H&L(O/F) test- Glucose	Acid, no gas
VP test	Negative
Indole	Positive
Acid from Glucose	Positive
Acid from Sucrose	Negative
Acid from Mannitol	Positive
Acid from Arabinose	Positive
Acid from inositol	Negative
Lysine decarboxylase	Positive
Ornithine decarboxylase	Positive
Arginine dihydrolase	Negative
Growt in 0% NaCl	Negative
„ 3% Na Cl	Positive
„ 6% NaCl	Positive
„ 8% NaCl	Positive
„ 10% NaCl	Negative
Growth at 42°C	Positive
Sensitivity to O/129 - 10 micro gram	Resistant
Sensitivity to O/129 -150 microgram	Sensitive
Gelatinase	Positive
Haemolysis on Wagatsuma Agar	β -haemolytic

3.2.6. *Staphylococcus aureus:-* Characterization, growth studies, pathogenicity and toxigenicity

3.2.6.1. Isolation

Staphylococcus aureus in fish and shellfish was enumerated as described in (3.2.1.6). Typical colonies were picked from B.P. plates, purified by streaking on NA plates and maintained on NA slants for further studies.

3.2.6.2. Biochemical characteristics of the cultures

The ability of the *Staphylococcus* cultures to produce Catalase, oxidase and coagulase, to ferment various sugars like Glucose, Maltose, Raffinose, Sucrose, Cellobiose, Salicin, Trehalose, Galactose, Arabinose, Xylose, Mannitol, Fructose, Lactose, Mannose, Melezitose, Ribose and to decarboxylate aminoacids like Lysine, histidine, and ornithine and dehydrolyse Arginine, were studied by standard methods (Salle 1954, USFDA, 2001, Surendran *et al*, 2003)

Using Hugh and Leifson's Oxidative /Fermentative medium, determined the mode of attack of sugars by the cultures. (Hugh & Leifson, 1953). The presence of cytochrome oxidase in the cultures was detected by the modified Kovac's test (Kovac's 1956). Catalase was detected by the evolution of gas when a drop of 30% hydrogen peroxide (V/V) was mixed with a speck of cultures on a clean slide. Pigmentation was observed on nutient milk agar plates after an incubation of 36±1°C for 48 hours (Surendran *et al*,2003).

3.2.6.3. Coagulase test

Bacto-Coagulase plasma EDTA (lyophilized rabbit plasma stabilized with EDTA) from Difco, USA was used for the test. A 3 ml plasma EDTA vial was reconstituted with 3 ml sterile distilled water, 0.5 ml each was pipetted into 6 coagulase test tubes (80 mm x10 mm glass tubes). Of the 6 tubes so obtained the 1^{st} was the reagent control, the 2^{nd} the media control and the rest for test. To the second tube 2 drops of sterile BHI was added.

To the rest, 2 drops of young *Staphylococcus* cultures in BHI broth (24 hr old) was pipetted in duplicate. The tubes were incubated in a serological water bath at 36±1°C for 4 hours and every 30-minute, observed for coagulation of the plasma.

A positive reaction was a clear clotting of the plasma in the culture inoculated tubes while both reagent and media control remain in the fluid state. Depending on the time taken for clotting, the pathogenicity of the *Staphylococcus* culture was graded as follows (Qualitative only).

Table. 3.2.6.3. Grading of pathogenicity based on coagulase test

Clotting at	Pathogenicity Grading
30 minutes or less	++++
31 –60 minutes	+++
61- 90 minutes	++
Above 90 minutes up to 4 hours	+
Others	-ve

3.2.6.4. Production of enterotoxins by *S.aureus* and toxin typing

All the isolates of *S.aureus* were tested for enterotoxin production. The cultures were inoculated in BHI broth and incubated at 37°C for 18-24 hours. After incubation they were centrifuged at 900x g for 20 minutes, at 4°C in a refrigerated centrifuge (Eppendorf 5804 R, Germany). The pellets were discarded. The supernatant was taken for assay.

Toxin production was tested and the toxin typing was done by Reverse Passive Latex Agglutination (RPLA) technique. Staphylococcal enterotoxin test kit (SET-RPLA, TD-900 Oxoid, UK) was employed for the toxin assay. The procedure described by the Oxoid was used. All the control reagents from the kit were reconstituted by adding 0.5 ml of the diluent provided. The latex reagents were shaken well before use to ensure uniform suspension. The V-bottom assay plates were arranged in such a way that each row

contains 8 wells. Each sample needed five such rows. Twenty-five microlitre of the diluent was dispensed in each well. To the first well 25 µl of the sample supernatant after centrifugation was added. Using a pipette, starting at the first well of each row, 25 microlitre from the first well was pipetted and added to the next well. This way of doubling dilution was performed for each of the five rows upto the seventh well. The last well was left to contain the diluent only. Latex sensitized with anti-enterotoxin A, B, C and D were added to each well in the first to fourth row respectively. To each well in the fifth row was added 25 microlitre of latex control. The plates were left on a vibration free surface at room temperature (28±2°C) for 16-24 hours. The plates after incubation were examined against a dark background for agglutination. Results classified as (+), (++) and (+++) are considered as positive. Results in the row of wells containing latex control should be negative. The last well in all rows was negative. If positive patterns were observed in some of these wells, the reaction was regarded as invalid.

3.2.6.5. Determination of lipolytic activity of *Staphylococcus* cultures

Lipolytic activities of the *Staphylococcus* cultures were determined based on their ability to hydrolyse tributyrin (a triglyceride) and the phospholipid present in egg yolk.

1. Tributyrin hydrolysis by cultures

Method of Collins and Lyne, (1985) was followed for testing the Tributyrin hydrolysis by *Staphylococcus* cultures. Tributyrin agar was used for determining the lipase activity. The pre- dried plates of tributyrin agar were spot inoculated with *Staphylococcus* cultures and incubated at 36±1°C for 24-48 hours. After incubation, the plates were observed for clearance around the bacterial colony due to lipolytic activity.

2. Phospholipase activity by cultures

Eggyolk agar was used for determining the phospholipase activity. Nutrient agar supplemented with 5% egg yolk emulsion was used as the medium. The pre-dried plates were spot inoculated with the *Staphylococcus* isolates and incubated at 36±1°C for 24-48

hours. After incubation, the plates were observed for opacity (a dull white precipitate) formed around the colony due to the formation of free fatty acids from phospholipids.

3.2.6.6. Determination of Urease activity (Harrigan and Mc Cance, 1976)

Christensen's urea agar was used for this test. The cultures were streaked on Urea agar slops and incubated at36±1°C for 24-48 hours. After incubation, the tubes were observed for a change of colour of the medium to pink (NH_3 formation). A pink colour indicated a positive reaction. Retention of the yellow colour of medium indicated a negative reaction.

3.2.6.7. Determination of Phosphatase activity (Collins and Lyne, 1985)

Nutrient agar with 0 .01% Phenolphathelene phosphate was used. The pre-dried plates were spot inoculated with *Staphylococcus* cultures and incubated overnight at 36±1°C for 18-24 hours. After incubation the plate was exposed to ammonia vapour. Pink or red colonies indicated free phenolphthalene released by phosphatase activity and hence a positive reaction.

3.2.6.8. Determination of Thermonuclease activity (Barry *et al*, 1973)

Nutrient agar with 1% DNA was used for this test. Pre-dried DNA agar plates were spot inoculated with the cultures and incubated at 36±1°C for 18-24 hours. After incubation the plates were acidified by flooding with one normal hydrochloric acid (1N HCl). Unaffected DNA will get precipitated. A zone of clearance around the colony indicated a positive reaction.

3.2.6.9. Determination of the proteolytic ability of the cultures (Harrigan and Mc Cance, 1976)

Proteolytic potential of *Staphylococcus* cultures was determined by studying the ability of the cultures to liquefy gelatin and hydrolyze milk protein (Casein).

1. Gelatin liquefaction by cultures

Frazier's Gelatin agar (modified) was used for determining the ability of the isolates to hydrolyse gelatin. Pre-dried plates of gelatin agar were spot inoculated with the cultures and incubated at 36±1°C for 18 - 96 hours. After incubation the plates were flooded with saturated

mercuric chloride solution. Unhydrolysed gelatin forms a white opaque precipitate with the mercuric chloride reagent. A clear zone around the colony indicated a positive reaction for gelatin hydrolysis.

2. Hydrolysis of milk protein (Casein) by cultures

The hydrolysis of milk protein (casein) by the bacterial cultures was determined using nutrient – skimmed milk (NASM) agar. Nutrient agar containing 5% skimmed milk powder was prepared. Pre-set and surface dried NASM agar was spot inoculated with the *Staphylococcus* cultures and incubated at 36±1°C for 48 hours. A positive proteolytic activity was visible as a clear zone of clearence around the colony.

3.2.6.10. Antibiotic Sensitivity test (Bauer and Kirby, 1966)

Antibiotic sensitivity of the *Staphylococcus* cultures were determined by the standard agar diffusion test (Bauer & Kirby, 1966) on Muller Hinton Agar (Oxoid, UK) using the following antibiotic disks Chloramphenicol (30mcg), Penicillin (2.5IU), Erythromycin (15mcg), Methicillin (5mcg), Novobiocin (30mcg) Tetracycline (30mcg), Oxytetracycline (30mcg), Neomycin (30mcg), Nalidixic acid (30mcg), Oxolinic acid(2mcg), Vancomycin(10mcg) & Sulphamethazole(25mcg). All the antibiotics discs were procured from Himedia, India. The sensitivity/ resistance was recorded as per the scheme of NCCLS (2005).

3.2.6.11. Classification of *Staphylococcus* up to species level (Harvey and Gilmour, 1985)

Based on the biochemical and cultural characteristics, the Staphylococcus cultures isolated from fish and fish fishery products were classified up to species level as per the scheme of Harvey and Gilmour (1985). The scheme is presented below (Table 3.2.6.11) for reference.

3.2.6.11. Classification of *Staphylococcus* up to species level (Harvey and Gilmour, 1985)

Staphylococcus sp	Test used													
	coagulase	Thermo	Haemolysins	Acetoin	Pigment	Sucrose	Trehalose	Mannitol	Cellobiose	Maltose	Mannose	Xylose	Phosphatase	Novobiocin
S.aureus	+	+	+	+	+	+	+	+	-	+	+	-	+	S
S.hysicus hysicus	-	+	-	-	-	+	+	-	-	-	+	-	+	s
S.hysicus chromogenes	-	-/+	-	-	+	+	+	+/-	-	+/-	+	-	+	S
S.simulans	-	-	-/+	-	-/+	+	-/+	+	-	-/+	-/+	-	-/+	S
S.intermedius	+	+	+	-	-	+	+	+	-	-	+	-	+	S
S.epidermidis	-	-	+/-	+	-	+	-	-	-	+	+	-	+	S
S.capitis	-	-	-	+/-	-	+	-	+	-	-	+	-	+/-	S
S.hominis	-	-	+/-	+/-	+/-	+	+	-	-	+	-	-	-	S
S.warneri	-	-	+/-	+	+	+	+	+/-	-	+	-	-	+/-	S
S.haemolyticus	-	-	+	+	+/-	+	+	+/-	-	+	-	-	+/-	S
S.cohini	-	-	+/-	+	+/-	-	+	+	-	+	+/-	-	-	R
S.saprophyticus	-	-	-	+	+/-	+	+	+	-	+	-	-	-	R
S.xylosus	-	-	-	+/-	+/-	+	+	+	-	+	+	+	+	R
S.sciuri sciuri	-	-	-	-	+	+	+	+	+	+/-	+/-	-	+	R
S.sciuri lentus	-	-	-	-	-	+	+	+	+	+/-	+	+/-	+/-	R

3.2.6.12. Growth kinetics and toxin production by *S.aureus* cultures

3.2.6.12.1 Effect of temperature on the growth and toxin production of *S. aureus*

Growth studies of the *S.aureus* cultures were done in Brain Heart Infusion broth (Difco, USA). BHI broth was prepared in 10 ml quantities in test tubes. A set of BHI tubes was inoculated with a known number of *S.aureus* cells grown in BHI broth (18-24 hours). The tubes were incubated in duplicate at 1±1°C, 8±1°C, 28±2°C , 37±1°C, 45±1°C, 56±1°C. All the tubes were incubated for a period varying from 7 days to 30 days.

Quantitative assessment of the growth of cultures at each temperature at regular intervals was done. This was done in the following way.

From each culture tube, 10 micro liters of the cultures were withdrawn aseptically at regular time intervals and plated on BP agar for enumerating the cells by the drop plate method of Miles *et al* (1938). Along with this procedure of withdrawal of the culture for enumeration of the cells, the enterotoxin production by the cultures was also monitored concurrently by the RPLA technique (ref: 3.2.6.3).

3.2.6.12.2. Generation time of Bacteria

The generation time of each *S.aureus* culture was calculated for each temperature of growth from the quantitative data obtained in the above experiments (3.2.6.12.1) using the following relation

$$\text{Generation time (g)} = \frac{t_2 - t_1}{3.32\,(\log_{10} n_2 - \log_{10} n_1)}$$

Where "n_1 is the number of cells at time "t_1 and n_2 number of cells at time t_2.

3.2.6.12.3. Effect of Salt on growth and toxin production of *Staphylococcus aureus*

For this study, Brain Heart Infusion (BHI) broth with different salt concentrations were prepared as follows. BHI broth (1050 ml) was divided into 7 lots of 150ml each. Sufficient quantities of Analar NaCl was added to get resultant sodium chloride concentration of 3%, 5%, 10%, 15%, 18%, and 20%. The pH of the medium was adjusted to pH 7.1 ±.1. The media were dispensed in 10ml volume in test tubes and sterilized at 15 lbs pressure for 15 minutes.

A selected number of *S.aureus* cultures were used. Cultures grown in 10ml BHI broth (0.5% NaCl) for 24 hours were harvested by centrifugation and the cells were washed with normal saline (0.85% NaCl) and then re suspended in normal saline. The suspension was then diluted with normal saline to a known opacity and number of cells/ ml in the dilution was determined by drop-plate method. The tubes were then incubated at 36±1°C for upto one month. From each tube with the above salt levels, 10 microlitre of the suspension was withdrawn at regular intervals and plated on BP agar to assess the growth (drop plate method Miles *et al,* 1938). Concurrently toxin production was determined by RPLA by withdrawing 250 microlitre of the culture broth at regular intervals.

3.2.6.12.4. Effect of pH on growth and toxin production of *Staphylococcus aureus*

BHI broth with different pH was used for the study. The pH of the medium was adjusted using 2N HCl and 5 N NaOH, to get resultant pH's of pH3, pH5, pH7, pH9 and pH12. Medium was dispensed in 10 ml quantities in test tubes. A selected number of cultures grown in BHI broth for 24 hours were harvested by centrifugation and the cells were resuspended in normal saline. The suspension was then diluted to a known opacity. A set of the above BHI broth tubes with the above pH values was inoculated with a

known number of cells and incubated at 36±1°C for one month. The growth and toxin production was estimated by withdrawing cultures at regular intervals as in 3.2.6.12.3.

3.2.6.12.5. Effect of water activity on growth and toxin production by *S.aureus*

BHI broth was used for the study. Water activity was adjusted to a known value using NaCl as the humectant. The value was measured with water activity meter (Lufft, Germany). Medium was dispensed in 10 ml quantities in test tubes. A selected number of cultures grown in BHI broth for 24 hours were harvested by centrifugation and the cells were re suspended in normal saline. The suspension was then diluted to a known opacity. A set of the above BHI broth tubes was inoculated with a known number of cells and incubated at 36±1°C for one month. The growth and toxin production was estimated by withdrawing cultures at regular intervals as in 3.2.6.12.3.

3.2.6.12.6. Effect of temperature on growth and toxin production by *S.aureus* in fresh raw shrimp meat

Fresh shrimp was procured from the retail fish markets in Cochin, packed in sterile polythene bags and brought to laboratory under ice. The shrimp was washed twice in potable water and peeled and deveined. Meat was washed with sterile distilled water and divided into different lots. The divided lots were inoculated with known number of *S.aureus* cells and stored at different temperatures, viz; 1±1°C, 8±1°C, 28±2°C, 37±1°C and (-) 20°C for periods ranging from 6 hours to 30 days. Samples were aseptically withdrawn at regular intervals and the growth of *S.aureus* was determined using spread plate method. Toxin production was determined by taking tissue samples at regular intervals. The tissue was homogenized with equal volume of saline and centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was taken, filtered through a 0.2-micron syringe filter. Assay was carried using SET-RPLA test kit as per 3.2.6.4 using the filtrate as sample.

3.2.6.12.7. Effect of temperature on growth and toxin production by *S.aureus* in cooked shrimp meat

Peeled and deveined shrimp meat was blanched at 90°C for 30 seconds and divided into different lots and inoculated with known number of *S.aureus* cells. The lots were then incubated at different temperatures as in the case of fresh raw shrimp. The growth and toxin production was determined as described in the case of fresh raw shrimp meat (3.2.6.12.6).

3.2.6.12.8. Effect of temperature on growth and toxin production by *S.aureus* in fresh fish meat

Fresh seer fish (*Scomberomorus spp*) collected from the local fish market were brought to laboratory in iced condition. The fish was washed well in potable water and surface was disinfected with alcohol. The skin was removed and tissue was separated aseptically. The meat was divided into different lots and a known number of *S.aureus* cells were inoculated in each lot and incubated at different temperatures (earlier discribed). The growth and toxin production of *S.aureus* was assessed as in the case of fresh raw shrimp meat (3.2.6.12.6).

3.2.6.12.9. Effect of temperature on growth and toxin production of *S.aureus* in cooked fish meat

Fresh seer fish collected from the market were brought to laboratory in iced condition. The fish was washed well in water, skin was removed and tissue was seperated. The meat was minced and 10 gms each was transferred to test tubes and sterilized by autoclaving at 15 lbs pressure for 15 minutes. After cooling the tubes overnight, a known number of *S.aureus* cells were inoculated in to each tube and incubated at different temperatures as the above. The growth and toxin production of the *S.aureus* cells were assessed as described earlier (3.2.6.12.6).

3.2.6.13. PCR amplification of toxigenic genes of *Staphylococcus*

3.2.6.13.1. PCR PRIMERS

PCR primers specific for Staphylococcus enterotoxin A,B,C and D were as per Johnson *et al* (1991) and are listed below.

Sl. No.	PRIMERS	SEQUENCE	SIZE (bp)
1	SEA1	TTG GAA ACG GTT AAA ACG AA	120
2	SEA2	GAA CCT TCC CAT CAA AAA CA	120
3	SEB1	TCG CAT CAA ACT GAC AAA CG	478
4	SEB2	GCA GGT ACT CTA TAA GTG CC	478
5	SEC1	GAC ATA AAA GCT AGG AAT TT	257
6	SEC2	AAA TCG GAT TAA CAT TAT CC	257
7	SED1	CTA GTT TGG TAA TAT CTC CT	317
8	SED2	TAA TGC TAT ATC TTA TAG GG	317

SEA- Staphylococcal enterotoxin A

SEB- Staphylococcal enterotoxin B

SEC- Staphylococcal enterotoxin C

SED- Staphylococcal enterotoxin D

3.2.6.13.2. DNA extraction

A speck of the *S.aureus* culture was inoculated in 3 ml BHI broth and incubated at 37°C for 18-24 hours. 1.5 ml of the culture was pipetted into a microfuge tube and centrifuged at 10000 rpm for one minute at 4°C in a refrigerated centrifuge (5804 R Eppendorf, Germany). The supernatant was decanted and 95 µl TE buffer (pH 8) and 5 µl lysozyme (Sigma) was added and incubated in a water bath at 37°C for 30 minutes. After incubation 900µl of double distilled water was added and heated in a water bath set at 90°C for 10 minutes for cell lysis. The cell lysate was then centrifuged at 10000 rpm for one minute at 4°C. The supernatant containing DNA was taken for PCR assay.

3.2.6.13.3. PCR assay

All the PCR conditions and reaction mixtures were standardized to get optimum results. PCR amplification was performed in 25 µl of total volume and included 1 µl of targeted DNA. The following concentrations of the various reagents were used. 1X PCR buffer (Biogene, USA), 0.2mM of dNTP mix, 1µ M of each primer pair, 0.05% (vol/vol) (Fermentas,USA) and 1 unit Taq polymerase (1U/ µl Biogene, USA). A single protocol was used through out and PCR was performed in a thermocycler (Eppendorf, Germany).

3.2.6.13.4. PCR Amplification condition

PCR amplification was carried out with the 1µl template DNA of the 50 *Staphylococcus* isolates by employing SEA1/SEA2, SEB1/SEB2, SEC1/SEC2 and SED1/ SED2 primers coding for genes of staphylococcal enterotoxin A, B,C and D respectively. The size of the amplicons was verified and optimal PCR conditions were determined in thermal cycler (Eppendorf, Germany). The mixture was assured to be mixed properly. Amplifications were performed in thermal cycler with the reaction mixture with the following conditions.

Initial denaturation	at 94°C for 4 minutes

30 cycles of

Denaturation	at 94°C for 2 minutes
Annealing	at 55°C for 2 minutes
Extension	at 72°C for 1 minute
Final extension	at 72°C for 7 minutes

3.2.6.13.5. Agarose Gel electrophoresis

PCR products were analyzed by agarose gel electrophoresis.

Procedure

Amplified gene products were subjected to electophoresis on a 2% agarose gel. Gel was prepared in TE buffer with ethidium bromide (0.5 µg/ml) in a gel tray. 10 µl

PCR product was mixed well with 2µl of gel loading buffer and the gel was run at 90 volts for 60-90 minutes. The sizes of the amplicons were determined by running 1µl of 100bp DNA ladder (Gene Ruler, Fermentas International, Germany) loaded simultaneously with the PCR products. The gels were visualized on UV- trans-illuminator at 360 nm and photographed using an UV gel documentation system (Alpha Innotech Corporation, USA).

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1. Microbial quality of fish, shellfish and fishery products

4.1.1. Bacterial quality of fresh fish from retail markets of Cochin

The lists of various finfish collected from the retail fish markets in and around Cochin are given in Table 3.1.1, under the materials and methods section (Chapter 3). A total of 51 samples, consisting of nine different species namely, thryssa (*Thryssa species*, 6 nos), pearl spot (*Etroplus suratensis,* 5 nos), oil sardine (*Sardinella longiceps,* 8 nos), Indian mackeral (*Rastrelliger kanagurta,* 8 nos), Tilapia (*Oreochromis mossambicus,* 5 nos), reef cod (*Epinephelus species,* 5 nos), rohu (*Labeo rohitha,* 4 nos), seer fish (*Scomberomorus commerson,* 6 nos) and Chinese pomfret(*Pampus chinensis,* 4 nos) were analyzed. Bacterial quality parameters like total plate count (TPC), Total coliforms (MPN), faecal coliforms (MPN), *E.coli* (MPN), faecal streptococci and *S.aureus* as well as the presence of pathogens like *Salmonella, Vibrio cholerae* and *Vibrio parahaemolyticus* are presented in Table. 4.1.1.

The total bacterial counts (TPC) of these nine species of fresh fish were more or less in the range of 10^5-10^6 cfu/g, except a few samples of Thryssa (*Thryssa species)* and pearl spot, where the total plate counts (TPC) were still higher at 10^7-10^8/g. Most of the samples had the presence of total Enterobacteriaceae (TEC) count in the range of 10^2-10^3 cfu/g. Total coliforms and faecal coliforms were detected in the range of 20-140^+ (MPN). Most of the faecal coliforms detected were found to be *E.coli*. The presence of faecal streptococci was also varying in the range of 10^2-10^4 cfu/g.

Except Thryssa species and Indian mackerel, certain number of the fish samples tested was found to carry *S.aureus* in the range of 100-1000/g. *V.parahaemolyticus* was not detected in any of the samples tested. *Salmonella* was detected only in eight out of fifty one samples. *V.cholerae* non-O1 was detected in 13 samples.

Table. 4.1.1. Bacterial quality of fresh fish from the retail markets of Cochin

Sl. No.	Sample*	TPC	Total Entero-bacteriaceae	Total Coliforms	Faecal Coliforms	*E.coli*	Faecal Streptococci	*S.aureus*	*V.cholerae*	*V.para-haemolyticus*	*Salmonella*
1	*Thryssa* Species (6)	$3.2x10^5$-$4.2x10^7$cfu/g	$2x10^2$-$3x10^2$cfu/g	20-140	20-140	45-110	100-$3x10^3$cfu/g	N.D	Present (1+ve)	N.D	N.D
2	Pearl Spot (5)	$2x10^5$cfu/g – $1.02x10^8$cfu/g	$3x10^2$-$2.2x10^4$cfu/g	45-140	45-140	25-45	$8x10^2$cfu/g - $3.9x10^4$cfu/g	0 -$2x10^3$cfu/g (2 +ve)	Present (5 +ve)	N.D	Present (2+ve)
3	Oil Sardine (8)	$4.5x10^5$ – $8x10^6$ cfu/g	$2x10^2$-$4x10^2$ cfu/g	110-140$^+$	110-140$^+$	30-140$^+$	$2x10^3$cfu/g- $4x10^3$ cfu/g	0-$1.4x10^3$ cfu/g (2+ve)	N.D	N.D	N.D
4	Indian mackeral (8)	$3.4x10^5$-$6x10^6$cfu/g	$3x10^2$-$4x10^2$cfu/g	45-140$^+$	30-140$^+$	30-140$^+$	$2x10^2$cfu/g- $4.5x10^2$cfu/g	N.D	Present (2+ve)	N.D	Present (2+ve)
5	Tilapia (5)	$5.5x10^5$-$2.6x10^6$ cfu/g	$3x10^2$-$3x10^3$cfu/g	45-140$^+$	25-140$^+$	25-140$^+$	$4.2X10^3$cfu/g – $5.5x10^3$cfu/g	0-$2x10^2$cfu/g (2+ve)	Present	N.D	Present (1 +ve)
6	Reef Cod (5)	$1.78x10^6$cfu/g- $3.2x\ 10^6$cfu/g	$2x10^2$-$3x10^2$cfu/g	110-140$^+$	110-140$^+$	45-110	$2x10^2$cfu/g $3x10^3$cfu/g	0- 10^3 (1+ve)	N.D	N.D	N.D
7	Seer Fish (6)	$2.87x10^5$ cfu/g- $3.86x10^5$cfu/g	$2.3x10^2$- $4x10^3$ cfu/g	110-140$^+$	110-140$^+$	45-140$^+$	$2.2x10^2$- $1.13x10^3$cfu/g	0- $2x10^3$ (1 +ve)	Present (4+ve)	N.D	Present (2 +ve)
8	Chinese Pomfret (4)	$5.4x10^4$cfu/g- $7.2x10^6$ cfu/g	$2.85x10^2$- $4.2x10^3$cfu/g	45-140	45-140	25-45	$2.5x10^2$- 10^3cfu/g	0-$2x10^2$ (1+ve)	N.D	N.D	Present (1+ve)
9	Rohu (4)	$5x10^4$cfu/g - $6x10^4$ cfu/g	$2.5x10^2$ – $3.2x10^3$ cfu/g	45-110	45-110	20-30	2 $x10^2$cfu/g $3.6x10^3$ cfu/g	0 - 10^3 (2 +ve)	N.D	N.D	N.D

*Numbers shown in paranthesis indicates the number of samples analyzed.

N.D. – Not detected

** Refer Table 3.1.1.1 for the scientific name of fishes.

Nambiyar and Iyer (1990) and Nambiar and Surendran (2003a) have made a detailed investigation on the microbial quality of fresh fishes sold in the retail markets of Cochin. They have found that 72% of the fresh fish were of poor quality based on total plate count (TPC) alone. In their study 21.6% of the fresh fish samples had a total plate count (TPC) excceding 10^7/g. If the criterion of *E.coli* counts were taken, then 72.3% of the fresh fishes examined by them were exceeding the limit of 20/g and 47.6% of the fish samples exceeded *E.coli* count of 20/g. Presence of *E.coli* is an indication of faecal contamination. Incidence of faecal streptococci is a supplementary proof for faecal contamination of fish. In the data presented in Table, 4.1.1 it is seen that faecal streptococci are invariably present in the orders of 100-1000/g in most of the samples of finfish from retail markets of Cochin. Nambiar and his colleagues have not reported faecal streptococci as a quality parameter. However in the present study the occurrence of faecal streptococci have been included and the results amply supported the conclusion derived from the presence of coliforms and *E.coli* in fish samples that the raw finfish from Cochin fish market are totally unhygienic for human consumption.

The investigation carried out by Varma *et al* (1989) reported the presence of *V.cholerae* O1 in only two samples out of the 1001 samples analyzed but they reported the presence of *Vibrio cholerae* non-O1 in 37.6% of the samples analyzed. Similar results were reported by Mathew *et al* (1988) who carried out the analysis of fresh fish samples for *Vibrio cholerae* in seafoods and environments of Mangalore. They reported the presence of *Vibrio cholerae* non-O1 in 16% of the samples. The present study also supports these findings, with a high incidence in 25.5% of the samples.

The assessment of the quality of fish landed at Cochin fisheries harbour by Lakshmanan *et al,* (1984) did not report any incidence of *Salmonella* in those samples. Nambiar and Iyer (1990) reported the incidence of *Salmonella* in 5.8% of the samples, Nambiar & Surendran, (2003) in 17.3% of the samples from the retail fish markets of

Cochin. In the present study also, *Salmonella* was invariably present in 15% of the total finfish samples analyzed.

USFDA, EU and BIS have stipulated maximum permissible limits for bacterial parameters in fresh fish for human consumption (USFDA, 2001; Surendran *et al*, 2003; BIS standards viz; IS: 4780 (1978), IS: 6032 (1971). Generally the upper limit fixed for TPC is below $5x10^5$ cfu/g, *E.coli*, 20/g, *S.aureus* 100/g and *Salmonella* and *Vibrio cholerae* to be absent in 25 grams. From the data presented in Table 4.1.1, it is clearly indicated that most of the finfish samples available in retail markets of Cochin were microbiologically unfit for human consumption based on these standards. Also,it is a matter of great concern that critical pathogens like *Vibrio cholerae* and *Salmonella* are present in at least 13 and 8 samples respectively. The unhygienic handling of the fresh fish samples in the retail markets has to be implicated as the major source of the presence of such pathogens and indicator bacteria.

4.1.2. Bacterial quality of fresh shrimp from the retail markets of Cochin

A total of 18 samples of shrimp consisting of white prawn (*Penaeus indicus*, 3 nos), tiger prawn (*Penaeus monodon,* 4 nos), kazhanthan (*Metapenaeus affinis,* 3 nos), poovalan (*Metapenaeus dobsoni,* 3 nos) and karikadi (*Parapaeneopsis stylifra,* 2 nos) and scampi (*Macrobrachium rosenbergii,* 3 nos) have been analyzed for their total plate count (TPC), total Enterobacteriaceae count (TEC), total coliforms (MPN), faecal coliforms (MPN), *E.coli*, faecal streptococci, *S.aureus* as well as pathogens like *Vibrio cholerae, Vibrio parahaemolyticus* and *Salmonella*. The results are presented in Table 4.1.2.

Out of the 18 samples, fifteen were marine prawns and three scampi samples were from fresh water. The total plate count (TPC) of almost all the samples was above 10^6cfu/g. In the case of three white shrimp samples and four tiger prawn samples, the

TPC exceeded the 10 million mark (10^7)/g. In two samples of *Metapenaeus affinis*, the total plate count was in the range of 10^5cfu/g.

The Enterobacteriaceae were invariably present in all the 18 samples analyzed, more or less in the range of 10^2-10^3 cfu/g. Total coliforms and faecal coliforms were 110-140^+ and a significant number of faecal coliforms were found to be *E.coli*. Faecal streptococci were also detected in all the samples varying from 10^2- 10^4cfu/g. Interestingly *S.aureus* were found only in two samples of the shrimp tested. Similar was the case with *Salmonella*. However *V.cholerae* non-O1 was found in four samples and *V.parahaemolyticus* in five samples of the eighteen samples of shrimp tested.

Quantitative studies on the bacterial quality of marine as well as fresh water shrimps have been reported by many authors. Some of these investigations were on freshly landed marine prawns. Surendran (1980) analyzed the bacterial quality of freshly caught prawns and reported a total bacterial count in the range of 10^3-10^5cfu/g in *P.indicus*, 10^2-10^6cfu/g in *M.affinis* and 10^3-10^6cfu/g in *M.dobsoni*. Antony *et al*, (2004) reported a total plate count in the range of $1.31x10^5$- 8.2 x 10^6cfu/g in shrimps landed in different fish landing centers of Tuticorin. They did not report the presence of any pathogens in these samples. Similar results were obtained by Anand *et al*, (2002). They also reported a very high bacterial load in the range of $1x10^4$-$1.21x10^8$cfu/g but they could not isolate any bacteria like *Vibrio cholerae* and *Salmonella* in these samples but they isolated *Vibrio parahaemolyticus* from few samples.

Jeyasekaran *et al*, (1990) reported that raw shrimps from Mangalore fish landing center had an *E.coli* count in the range of 60-240. Antony *et al*, (2004) reported an *E. coli* count in the range of 26-542/g. In the present study, the *E. coli* count varied between 20-110^+/g. Anand *et al*, (2002) reported a total coliform count in the range of 28-1100 MPN/g, and faecal coliform count in the range of 11-1100MPN/g and a low *E.coli* count. The recorded *E.coli* count was in the range of 10 cfu/g in shrimps landed in Tuticorin fishing harbour. The data presented in Table 4.1.2 shows an *E.coli* count in the range of

4.1.2. Bacterial quality of fresh shrimp from the retail markets of Cochin

Sl. No.	Sample*	TPC	Total Entero-Bacteriaceae	Total Coliforms	Faecal Coliforms	*E.coli*	Faecal Streptococci	*S.aureus*	*V.cholerae*	*V.para-haemolyticus*	*Salmonella*
1	White shrimp	$2.3x10^6$cfu/g	$2x10^2$cfu/g	140	140	20	$8.5x10^4$cfu/g	N.D	N.D	N.D	N.D
2	White shrimp	$2.31x10^7$cfu/g	$5.5x10^2$cfu/g	140	140	140	$3x10^3$cfu/g	N.D	N.D	N.D	N.D
3	White shrimp	$1.85x10^7$cfu/g	$2x10^3$cfu/g	140	140	140	$6.4x10^4$cfu/g	$4x10^2$cfu/g	N.D	Present	N.D
4	Tiger prawn	$4.18x10^7$cfu/g	$1.7x10^3$cfu/g	140	140	140	$3x10^2$cfu/g	N.D	N.D	N.D	N.D
5	Tiger prawn	$2.6x10^7$cfu/g	10^3cfu/g	140	140	110	$1.8x10^3$cfu/g	N.D	N.D	N.D	Present
6	Tiger prawn	$4.74x10^7$cfu/g	$7.3x10^3$cfu/g	140	140	95	$1.3x10^4$cfu/g	N.D	Present	N.D	N.D
7	Tiger prawn	$3.8x10^7$cfu/g	$2x10^3$cfu/g	140	140	110	$3.8x10^4$cfu/g	10^3cfu/g	N.D	N.D	N.D
8	Poovalan	$2.32x10^6$ cfu/g	$6x10^2$cfu/g	140	110	95	$2x10^2$cfu/g	N.D	N.D	Present	N.D
9	Poovalan	$1.8x10^6$ cfu/g	$5x10^2$cfu/g	140	110	110	$8.2x10^3$cfu/g	N.D	Present	Present	N.D
10	Poovalan	$4x10^6$ cfu/g	$2.2x10^2$ cfu/g	140	140	110	$2x10^4$cfu/g	N.D	N.D	N.D	N.D
11	Karikadi	$5.8x10^6$cfu/g	$2.8x10^2$cfu/g	140^+	140^+	140^+	$4.8x10^4$ cfu/g	N.D	Present	N.D	N.D
12	Karikadi	$2.8x10^6$cfu/g	$1.16x10^3$cfu/g	140^+	140^+	140^+	$3.5x10^4$cfu/g	N.D	N.D	N.D	N.D
13	Scampi	$3.4x10^6$cfu/g	$2.3x10^3$cfu/g	140	140^+	110	$1.34x10^4$cfu/g	N.D	Present	N.D	Present
14	Scampi	$6.5x10^6$cfu/g	$1.8x10^2$cfu/g	110	95	45	$6.17x10^2$cfu/g	N.D	N.D	N.D	N.D
15	Scampi	10^6cfu/g	$2.9x10^2$cfu/g	110	110	95	$3.92x10^2$cfu/g	N.D	N.D	N.D	N.D
16	Kazhanthan	$5x10^5$cfu/g	$2.4x10^2$cfu/g	140	140	45	$9.6x10^3$cfu/g	N.D	N.D	N.D	N.D
17	Kazhanthan	$5.85x10^5$cfu/g	$3.2x10^3$cfu/g	140	110	95	$8.4x10^2$cfu/g	N.D	N.D	Present	N.D
18	Kazhanthan	$1.18x10^6$cfu/g	$1.84x10^3$cfu/g	140	140	30	$6.2x10^2$cfu/g	N.D	N.D	Present	N.D

* Refer Table 3.1.1.2. for the scientific name of shrimps tested.

20-110^{+}MPN/g. This indicates that all shrimp samples collected from the retail markets were invariably contaminated with *E.coli*. Jeyasekaran *et al,* (1990) reported a very low *E.coli* count in samples from the Mangalore fishing harbour of Karnataka. Surendraraj *et al,* (2005) reported an *E.coli* count in the range of 2 log cfu/g - 5 log cfu/g in samples from the retail markets of Cochin. The variation in *E.coli* counts can be attributed to the seasonal variation and degree of faecal contamination of the different fishing areas and fish contact surfaces.

Anand *et al,* (2002) and Antony *et al,* (2004) reported a very high *S.aureus* count in the range of $2.12x10^3$cfu/g - $1.36x10^3$cfu/g and $6x10^4$cfu/g - $8.85x\ 10^5$cfu/g, in the samples collected from Tuticorin fishing harbour and fish landing center respectively. The very high count at this level are significant in the sense that a *S.aureus* count above 10^6/g can produce detectable levels of toxin that can cause food intoxication. In the present study the incidence of *S. aureus* was detected in only three samples and that too was in the range of 10^2-10^3cfu/g.

Bhaskar *et al,* (1998) reported the prevalence of *Vibrio cholerae* in cultured shrimp *Penaeus monodon.* Anand *et al,* (2002) and Antony *et al,* (2004) could not isolate any *Vibrio cholerae* from shrimp landed in Tuticorin fishing harbour and fish landing centers. In the present study *V.cholerae* non-O1 was confirmed in 22.22% of the samples analyzed.

Different authors reported the incidence of *Salmonella* in shrimp samples collected from different places. Venkateswaran *et al,* (1985) reported the incidence of *Salmonella* in 24% of the samples from the Parangipettai landing centre. Hatha and Laksmanaperumalsamy, (1997) could isolate *Salmonella* from 17.4% of the samples from Coimbatore fish market. A similar trend was observed in the present study also with an incident rate of 16.6% in shrimp samples from the retail markets of Cochin.

Vibrio parahaemolyticus has been isolated from shrimp samples by different authors. Since this bacterium has got the ability to utilize chitin it is usually found

attached on the surface of crustaceans like shrimps and crabs. Bandekar *et al,* (1982) isolated *V.parahaemolyticus* from the retail markets of Bombey. Karunasagar *et al,* (1985) reported the association of *V.parahaemolyticus* in prawns caught from off Mangalore coast. Sanjeev and Stephan (1993) isolated *V.parahaemolyticus* from different shrimp samples like *Parapaeneopsis stylifera* (83.33%), *Metapenaeus dobsoni /Metapenaeus affinis* (75%), *Metapenaeus affinis* (72.72%), *Penaeus indicus* (61.11%) from the markets of Cochin. In the present study also *V.parahaemolyticus* was detected in 27.5% of the samples.

In the case of ocean fresh prawns as reported by Surendran, (1980) the bacterial loads on shrimps were in a low range. However in the case of shrimps from markets of Mangalore, Mumbai and Cochin, higher bacterial counts have been recorded. In the present study, all the samples of shrimps from local markets were though apparently fresh, harboured very high bacterial load viz; 10^6-10^7 cfu/g. Similar finding of high bacterial load has been found from other samples from markets as already discussed. Also incidence of pathogens like *Salmonella* and *V.parahaemolyticus* were also found in shrimp samples collected from Parangipettai, Mangalore and Bombay. From the microbiological quality stand point, the shrimp from retail markets of Cochin are not meeting the quality parameters fixed by the authorities concerned (USFDA, EU, BIS).

4.1.3. Bacterial quality of fresh whole crab from the retail markets of Cochin

A total of ten samples of fresh whole crabs consisting of 5 samples of sea crab (*Portunus pelagicus*) and 5 samples of mud crab (*Scylla serrata*) have been investigated for their bacterial quality. Table 4.1.3 gives the results. Seven crab samples carried a total bacterial load in the range of 10^5cfu/g while two samples had a bacterial load of 10^6cfu/g and in one sample the total plate count exceeded 10^7cfu/g. The total Enterobacteriaceae count was in the range of 10^2 to 10^3 in the case of eight samples while the total Enterobacteriaceae was above 10^4 and above in the remaining two samples.

4.1.3. Bacterial quality of fresh whole crabs from the retail markets of Cochin

Sl. No.	Sample	TPC	Total Entero-Bacteriaceae	Total Coliforms	Faecal Coliforms	*E.coli*	Faecal Streptococci	*S.aureus*	*Vibrio Cholerae*	*V.para-haemolyticus*	*Salmonella*
1	Sea crab	$1.66x10^7$cfu/g	$2.1x10^5$cfu/g	140	140	30	$1.39x10^4$cfu/g	$2x10^2$cfu/g	N.D	N.D	N.D
2	Sea crab	$9.5x10^5$ cfu/g	$2.4x10^3$cfu/g	110	110	25	$3x10^2$cfu/g	N.D	N.D	N.D	N.D
3	Sea crab	$3.8x\ 10^5$cfu/h	10^2cfu/g	110	110	25	$4.2x10^2$cfu/g	N.D	N.D	Present	N.D
4	Sea crab	$6.5x10^5$cfu/g	$3.4x10^3$cfu/g	110	140	30	100	N.D	N.D	Present	N.D
5	Sea crab	$5.3x10^5$cfu/g	$4.5x10^2$cfu/g	140	140	45	$4x10^2$cfu/g	N.D	N.D	N.D	N.D
6	Mud crab	$5.5x10^5$cfu/g	$3x10^4$cfu/g	110	110	30	$3x10^2$cfu/g	N.D	N.D	N.D	N.D
7	Mud crab	$2.76x10^5$cfu/g	$2x10^2$cfu/g	140	140	45	10^3cfu/g	$4x10^3$cfu/g	Present	Present	N.D
8	Mud crab	$5.5x10^5$cfu/g	$3.6x10^3$cfu/g	140	110	25	$2.5x10^2$cfu/g	N.D	N.D	Present	N.D
9	Mud crab	$8.5x10^6$cfu/g	$4.5x10^2$cfu/g	140	140	45	$4.8x10^2$ cfu/g	N.D	N.D	N.D	N.D
10	Mud crab	$2.8x10^6$cfu/g	10^3cfu/g	110	110	30	$2.1x10^3$cfu/g	N.D	N.D	N.D	N.D

* Refer Table 3.1.1.3 for scientific name of crabs tested.

Invariably all the samples carried coliform bacteria in the range of 110-140^{+}. A sizable percentage of faecal coliforms were found to be *E.coli*. Faecal streptococci was present in all the samples in the order of 10^2- 10^4 cfu/g. *S.aureus* were detected only in two samples, while *V.parahaemolyticus* was present in 4 out of the ten samples. *Vibrio cholerae* non-O1 was detected in one sample but *Salmonella* was not detected in any of the samples.

Studies on the bacterial quality of crabs are very scanty. In India, Kumar and Dube, (1990) has studied the total halotolerant and limnotolerant bacterial counts of crab (*Neptunus sanguinolentus*) and found a total plate count (TPC) in the range of 10^5/g. Hatha and Lakhmanaperumalsamy, (1997) have investigated the quality of sea crab (*Portunus pelagicus*) and mud crab (*Scylla serrata*) from the retail markets of Coimbatore, Tamil Nadu. They have found that the presence of *Salmonella* in sea crab and Mud crab. However they have not reported on other bacterial parameters in those samples. Lee and Pfeifer, (1975) reported a bacterial count in the range of 10^4-10^7cfu/g in the Dunges crab (*Cancer magister*) from the retail markets of Oregon (U.S.A).

The present study on the bacterial quality of whole crab from retail markets appears to be a pioneer work. A detailed microbiological study has been done on all the bacterial quality factors including the presence of critical pathogens and toxigens.

Based on the data presented it could be seen that the bacterial quality of fresh whole crab from the retail markets of Cochin are not fit for human consumption based on approved quality parameters.

4.1.4. Bacterial quality of fresh cephalopods from the retail markets of Cochin

Four samples of fresh squid (*Loligo duvacelli*) and four samples of fresh cuttle fish (*Sepia pharaonis*) from the retail markets of Cochin were analyzed for their bacterial quality including the presence of pathogens. The data are presented in Table.4.1.4.

4.1.4.Bacterial quality of fresh cephalopods from the retail markets of Cochin.

Sl No	Sample	TPC	TEC	Total Coliforms	Faecal Coliforms	*E.coli*	Faecal Streptococci	*S. aureus*	*V.cholerae*	*V.para-haemolyticus*	*Salmonella*
1	Squid Whole	$1x10^6$cfu/g	10^3cfu/g	140	140	110	$9x10^2$cfu/g	$3.4x10^3$cfu/g	N.D	N.D	N.D
2	Squid whole	$1.5x10^6$cfu/g	$2.8x10^2$cfu/g	140	110	N.D	N.D	N.D	N.D	N.D	N.D
3	Squid Whole	$8x10^6$ cfu/g	$5.2x10^3$cfu/g	140^+	140^+	110	$1.3x10^4$ cfu/g	N.D	N.D	N.D	N.D
4	Squid Whole	$9x10^5$cfu/g	$5x10^2$cfu/g	140^+	140^+	95	$3x10^4$cfu/g	$2x10^2$ cfu/g	N.D	N.D	N.D
5	Cuttle-fish whole	$1.4x10^7$cfu/g	$1.1x10^2$cfu/g	140	140	N.D	N.D	$3.8x10^4$cfu/g	N.D	N.D	N.D
6	Cuttle-fish whole	$5.8x10^6$cfu/g	10^3cfu/g	110	110	N.D	$3.4x10^3$cfu/g	N.D	Present	N.D	N.D
7	Cuttle-fish whole	$9x10^6$cfu/g	10^2cfu/g	140	110	95	$4.5x10^2$cfu/g	N.D	N.D	N.D	N.D
8	Cuttle-fish whole	$2x10^7$cfu/g	$3x10^3$cfu/g	140	110	110	$2.5x10^2$cfu/g	$2.8x10^4$cfu/g	N.D	N.D	N.D

The total plate counts (TPC) of whole squid were in the range of 10^5-10^6cfu/g while those of cuttle fish were in the range of 10^6-10^7cfu/g. Total Enterobacteriaceae counts in both squid and cuttle fish ranged between 10^2-10^3 cfu/g. Total coliforms and faecal coliforms were in the range of 110-140$^+$ of MPN. Except in one sample of squid and two samples of cuttle fish, the faecal coliforms were found to be *E.coli*. Faecal streptococci were detected in 6 samples in the range of 10^2-10^4 cfu/g. *V.cholerae* non-O1 were detected only in one out the 8 samples while *Vibrio parahaemolyticus* and *Salmonella* were not detected in any of the samples

So far, no work has been reported on the bacterial quality of fresh cephalopods namely squid and cuttle fish; so no comparison of our work could be made with already published data.

The USFDA has stipulated a total plate count of $2x10^5$ cfu/g for fresh cephalopods meant for human consumption. Based on this parameter on total plate count (TPC) as well as parameters on *E.coli*, *S.aureus* the fresh cephalopods marketed in Cochin are of poor quality. However the absence of critical pathogens on these cephalopods on retail market shows that they are comparatively safer with other shellfishes available in retail trade.

4.1.5. Bacterial quality of fresh green mussel (*Perna viridis*) from the farms and markets of Calicut

Eighteen samples of fresh green mussel (*Perna viridis*), ten collected directly from farm and eight wild samples from the retail markets of Calicut were studied for their bacteriological quality in detail and the data are presented in Table 4.1.5.

The total plate count (TPC) of the farmed samples were more or less in the range of 10^4-10^5 cfu/g while those of wild green mussels from retail markets were very high, mostly in the range of 10^6- 10^7cfu/g. The total Enterobacteriaceae (TEC) count of the farmed samples were in the range of 10^2-10^4cfu/g where as the total Enterobacteriaceae count of wild green mussel were mostly in the 10^2/g range. The farmed samples had

4.1.5. Bacterial quality of fresh green mussel (*Perna viridis*) from the farm & markets of Calicut.

Sl* NO	TPC	Total Entero-bacteriaceae	Total Coliforms	Faecal coliforms	*E.coli*	Faecal streptococci	*S.aureus*	*V.cholerae*	*V.para haemolyticus*	*Salmonella*
1	$6.2x10^4$cfu/g	$2x10^2$/g	95	45	N.D	10^3cfu/g	N.D	N.D	N.D	N.D
2	$2.1x10^4$cfu/g	$1.6x10^4$cfu/g	95	95	45	$2x10^2$cfu/g	N.D	Present	Present	N.D
3	$1.83x10^5$ fu/g	$5x10^2$cfu/g	2.5	2.5	0.4	$3x10^3$cfu/g	N.D	N.D	N.D	Present
4	$7.9x10^4$cfu/g	$3.1x10^3$cfu/g	25	25	25	$9.7x10^3$ fu/g	N.D	N.D	N.D	N.D
5	$2.12x10^5$ fu/g	$4x10^3$cfu/g	140^+	4.5	45	$2.8x10^3$cfu/g	N.D	N.D	N.D	N.D
6	$1.24x10^5$cfu/g	$4.2x10^3$cfu/g	25	25	25	$5.4x10^3$cfu/g	N.D	N.D	N.D	N.D
7	10^5cfu/g	$2.1x10^2$cfu/g	140	110	95	$2.3x10^2$cfu/g	N.D	Present	Present	N.D
8	$7.6x10^4$cfu/g	100/g	95	45	45	$3.8x10^2$cfu/g	N.D	N.D	N.D	N.D
9	$6.9x10^5$cfu/g	$4.2x10^2$cfu/g	140	110	95	$5x10^2$cfu/g	N.D	Present	N.D	N.D
10	$1.8x10^5$cfu/g	$6x10^2$cfu/g	110	110	45	$2x10^2$cfu/g	N.D	N.D	Present	N.D
11	$2.45x10^7$cfu/g	$8.4x10^3$cfu/g	140	140	140	200cfu/g	N.D	N.D	N.D	N.D
12	$3.2x10^6$cfu/g	$2x10^2$cfu/g	25	25	25	200cfu/g	N.D	N.D	N.D	N.D
13	$4.6x10^5$cfu/g	$2x10^2$cfu/g	95	95	45	$2.5x10^2$cfu/g	N.D	N.D	N.D	N.D
14	$5.5x10^5$cfu/g	$6x10^2$cfu/g	140	110	110	10^3cfu/g	N.D	N.D	N.D	Present
15	$1.28x10^6$cfu/g	$2.5x10^2$cfu/g	140	140	110	$2x10^2$cfu/g	N.D	Present	N.D	N.D
16	$1.09x10^7$cfu/g	$4.5x10^2$cfu/g	140	110	110	$6.2x10^2$cfu/g	N.D	N.D	N.D	Present
17	$3.2x10^6$cfu/g	$5x10^2$cfu/g	140	110	110	$9.2x10^2$cfu/g	N.D	N.D	Present	N.D
18	$1.65x10^6$cfu/g	$2.4x10^2$cfu/g	140	110	95	$3x10^2$cfu/g	N.D	N.D	Present	N.D

* Samples 1-10 are farmed green mussel directly from farm and 11-18 green mussel from retail market

comparatively lower number of total coliforms, faecal coliforms and *E.coli*, while the wild samples had total coliforms, faecal coliforms and *E.coli* at higher levels. Faecal streptococci were present in all the samples in the range of 10^2 - 10^3 cfu/g. *S.aureus* were not detected in any of the samples. *V.cholerae* non-O1 was found in four out of the 18 samples, *V.parahaemolyticus* in 5 samples and *Salmonella* in three samples.

From India, only a few studies have been reported on bacterial quality of mussels. Surendran *et al*, (1986) has reported a total plate count in the range of 10^3-10^6cfu/g in the wild mussel samples collected from the shallow sea off Calicut. They have recorded the total coliforms in the range of 36-2150/g; faecal coliforms and *E.coli* were in the range of 0-115 MPN/g. Faecal streptococci counts were in the range of 30-1.8x10^3cfu/g. Another study by Raveendran *et al,* (1990) have investigated the occurrence of faecal indicator bacteria and pathogens in the brown mussel (*Perna indica*) from the south west coast of India and found the presence of indicator bacteria and *Salmonella* in some of the samples. Iyer and Varma, (1987) and Gore *et al,* (1992) have reported the incidence of *Salmonella* in mussel meat collected from Calicut fish market and Mahe fish market respectively.

It can be incidentally stated that the present study on the bacterial quality of fresh green mussel can be considered as unique, particularly being the first on the bacterial quality of farmed green mussel directly collected from farms of Calicut. From the microbiological stand point the mussels whether farmed or wild are reservoirs of faecal bacteria. Hence these samples are not very safe for direct consumption. They need scientifically planned depuration in seawater in order to improve the food quality and safety.

4.1.6. Bacterial quality of black clam (*Villoritta cyprinoides*) from the retail markets of Cochin

Eleven samples of raw clam meat extracted directly from the whole clams and nine samples of boiled and shucked clam meat sold in the retail markets of Cochin were

4.1.6. Bacteriological quality of clams (*Villoritta cyprinoides*) from the retail markets of Cochin

Sl No*	TPC	TEC	Total Coliforms	Faecal Coliforms	*E.coli*	Faecal Streptococci	*S.aureus*	*V.cholerae*	*V.para-haemolyticus*	*Salmonella*
1	$1.91x10^5$cfu/g	$4x10^2$cfu/g	110	110	N.D	N.D	N.D	Present	N.D	N.D
2	$1.68x10^7$cfu/g	$1.8x10^5$cfu/g	140	140	110	$1.8x10^5$cfu/g	N.D	Present	N.D	N.D
3	$4.56x10^5$cfu/g	$3.2x10^3$cfu/g	140	95	45	$1.2x10^3$cfu/g	N.D	N.D	N.D	N.D
4	$5.9x10^4$cfu/g	$2.6x10^2$cfu/g	95	2.5	2.5	$1.15x10^3$cfu/g	N.D	N.D	N.D	N.D
5	$2x10^7$cfu/g	$2x10^2$cfu/g	140^+	140^+	140^+	$2.6x\ 10^4$cfu/g	N.D	N.D	N.D	N.D
6	$2.9X10^4$cfu/g	$1.15x10^2$cfu/g	140^+	140^+	140^+	$9x10^2$cfu/g	N.D	N.D	N.D	N.D
7	$6.5x10^7$ cfu/g	$2x10^2$cfu/g	140	140	140	$2.2x10^5$cfu/g	N.D	N.D	N.D	N.D
8	$1.08x10^5$cfu/g	$2.85x10^3$cfu/g	9.5	4.5	45	$2.2x10^3$cfu/g	N.D	N.D	N.D	N.D
9	$4.65x10^4$cfu/g	$7.45x10^3$cfu/g	140^+	140^+	45	$5.2x10^3$cfu/g	N.D	Present	N.D	N.D
10	$1.5X10^5$cfu/g	$2x10^2$cfu/g	110	110	20	200	N.D	N.D	N.D	N.D
11	$1.22x10^5$cfu/g	$1.1x10^3$cfu/g	140^+	140^+	140^+	$3.5x10^4$cfu/g	N.D	Present	N.D	N.D
12	$4.9x10^7$cfu/g	$6x10^3$cfu/g	140	140	95	$7.4x10^3$cfu/g	N.D	Present	N.D	N.D
13	$1.1x10^8$cfu/g	$1.6x\ 10^3$cfu/g	140	140	110	$1.8x10^4$cfu/g	$6x10^2$	N.D	N.D	N.D
14	$1.8x10^6$cfu/g	$4.3x10^3$cfu/g	140	140	140	$6.2x10^3$cfu/g	N.D	N.D	N.D	N.D
15	$2.7x10^7$cfu/g	$3.2x10^3$cfu/g	140	110	140	$2.6x`10^3$cfu/g	N.D	N.D	N.D	Present
16	$5x10^6$cfu/g	$3.8x10^2$cfu/g	140	140	110	$7.5x10^3$cfu/g	$5x10^2$	N.D	N.D	N.D
17	$4.2x10^7$cfu/g	$3x10^4$cfu/g	140	140	110	$5x10^2$cfu/g	N.D	Present	Present	N.D
18	$6.3x10^6$cfu/g	$6x10^2$ cfu/g	140	140	95	$4.5x10^2$cfu/g	N.D	Present	Present	N.D
19	$4.8x10^7$cfu/g	$1.1x10^3$cfu/g	140	110	95	$1.8x10^3$cfu/g	$3.5x10^3$cfu/g	Present	Present	N.D
20	$2.9x10^7$cfu/g	$2.84x10^3$cfu/g	110	110	45	$2.2x10^3$cfu/g	$5.5x10^2$cfu/g	N.D	Present	Present

- Samples 1-11, raw clam meat extracted directly from whole clams.
- Samples 12-20 boiled and shucked clam meat sold in retail market.

investigated for their bacterial quality including the presence of pathogens. The data are presented in Table 4.1.6.

Except two samples the total plate counts of all the raw clam meat were in the range of 10^4-10^6cfu/g, while the shucked clam meat from the market carried a bacterial load in the range of 10^6-10^8 cfu/g. The total Enterobacteriaceae count was more or less in the range of 10^2-10^3cfu/g. Total coliforms and faecal coliforms were mostly in the range of 110-140$^+$(MPN) while *E.coli* was recorded in a lower range. Faecal streptococci were found in nineteen out of the twenty samples that too in the range of 10^2 10^5cfu/g. *S.aureus* was found only in 4 out of the twenty samples while *Salmonella* was detected in only 2 samples. *V.cholerae* non-O1 was detected in 8 out of 20 samples while only 4 samples carried *Vibrio parahaemolyticus.*

Many workers in India and abroad have reported bacterial quality of clam meat. Vijayan *et al,* (1982) had made a detailed investigation on the bacterial quality of clam in the retail markets. They reported a total plate count (TPC) in the range of 10^6 cfu/g, total Enterobacteriaceae count $6.3x10^3$, *E.coli* $3x10^3$, and faecal streptococci $8.5x10^3$cfu/g. Gopakumar *et al,* (1993) and George and Gopakumar, (1995) have also reported the bacterial quality of clam meat. They reported a total plate count in the range of 10^5cfu/g, total coliforms in the range 103-172/g and faecal streptococci count in the range 120-154 cfu/g. 5.4% of the clam samples carried *Salmonella.* Surendraraj *et al,* (2005) have found a very high bacterial load in boiled and shucked clam meat from the retail markets of Cochin. Chai *et al,* (1990) have studied the bacterial quality of clam (*Mya arenaria*) from Chespeake Bay. The average total plate counts were in the range of 10^4/g, total coliforms in the 1500-6300/g, faecal coliforms in the 30-62/100g and *E.coli* less than 27/100g. Ekanem and Adegoke, (1995) has found a total plate count in the range of $8.1x10^7$/100g in the case of West African clam (*Egeria radiata,* Lamarch).

The data presented in Table 4.1.6 clearly indicated that the clam meat, raw or shucked available in the retail markets of Cochin were not microbiologically safe. This

points to the necessity for depuration of clams before shucking for consumption for human food. In the present study bacterial quality of shucked clam meat were much inferior than raw clam meat extracted directly from shucked clam meat.

The process of shucking of clam meat involves the boiling of clam meat for short term and agitating boiled clam meat in bamboo baskets with big holes to allow the meat alone to pass through. One should naturally expect that the bacterial quality of shucked meat should be superior to the raw whole clam meat, but in actual practice reverse is the case, because the shucked meat come in contact with the contaminated surface of agitating baskets as well as the hands of the handler. This could be the reason for the poor microbial quality of the shucked clam meat.

4.1.7. Bacterial quality of oysters from the farms of Kollam

The edible oyster (*Crassostrea madrasensis*) is not usually available in the retail fish markets of Cochin. So for this study, they were procured from the edible oyster farms of Kollam district of South Kerala where the farming operations are in small way for utilizing the technical know-how from Central Marine Fisheries Research Institute (Ernakulam).

Nine fresh and live samples of edible oyster (*Crassostrea madrasensis*) were procured over a period of 2 years from oyster farms at Kollam. Samples were analyzed for their bacterial quality including the presence of pathogens like *V.cholerae*, *V.parahaemolyticus* and *Salmonella*. The results are presented in Table 4.1.7. For the purpose of microbiological analysis, the oyster meat was aseptically extracted from the oyster and no boiling step was involved in the process so the results are a direct reflection of the microbial profile of the oyster meat. The total plate count (TPC) of all the samples except samples 2 and 5 were in the range of 10^4-10^5 cfu/g while the total plate count of samples 2 and 5 were in the 10^6cfu/g range.

4.1.7. Bacterial quality of edible oyster (*Crassostrea madrasensis*) from farms of Kollam

Sl No	TPC	Total Entero-bacteriaceae	Total Coliforms	Faecal Coliforms	*E.coli*	Faecal Streptococci	*S.aureus*	*V.cholerae*	*V.para-haemolyticus*	*Salmonella*
1.	$1.55x10^4$cfu/g	$1.7x10^3$cfu/g	140	140	95	$2x10^3$cfu/g	N.D	N.D	N.D	N.D
2.	$9x10^6$cfu/g	10^2cfu/g	140	45	25	$3x10^2$cfu/g	$4x10^4$cfu/g	N.D	N.D	N.D
3.	$1.6x10^4$cfu/g	$2.5x10^2$cfu/g	25	25	25	100	N.D	N.D	N.D	N.D
4.	$6.4x10^4$cfu/g	$2x10^4$cfu/g	25	25	2.5	$2x10^2$cfu/g	$2x10^2$cfu/g	N.D	N.D	N.D
5.	$6x10^6$ cfu/g	$2.2x10^4$cfu/g	45	45	45	200	N.D	N.D	N.D	N.D
6.	$1.8X10^4$cfu/g	$6x10^2$cfu/g	140	140	45	$3x10^3$cfu/g	N.D	N.D	N.D	N.D
7.	$5.7x10^4$cfu/g	$5x10^2$cfu/g	25	9.5	9.5	$4.35x10^3$cfu/g	N.D	N.D	N.D	N.D
8.	$4.5x10^4$cfu/g	$4.5x10^2$cfu/g	45	45	45	$1.17x10^2$cfu/g	N.D	N.D	N.D	N.D
9.	$1.27x10^5$cfu/g	$3.6x10^3$cfu/g	4.5	2.5	2.5	$5.6x10^2$cfu/g	N.D	N.D	N.D	N.D

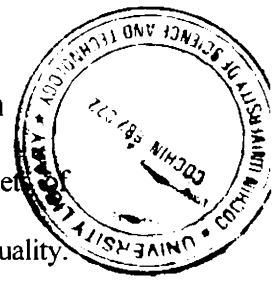
Total Enterobacteriaceae count (TEC) was between 10^2-10^4 cfu/g. The coliforms were comparatively less. Most of the samples had total coliforms, faecal coliforms and *E.coli* in the range between 2.5- 45MPN/g. Faecal streptococci were in the range of 10^2 to 10^3 cfu /g. *S.aureus* were present only in 2 out of the 9 samples while *V.cholerae*, *Salmonella* and *Vibrio parahaemolyticus* were not detected in any of the samples.

The data presented in Table 4.1.7 definitely indicates that the edible oyster samples from the farms of Kollam harboured less number of bacteria compared with other molluscan shellfish analyzed. Further it is very heartening to note that no critical pathogens namely *V.cholerae*, *V.parahaemolyticus* and *Salmonella* were detected in the muscle of the edible oyster.

From India, much work has not been reported in the microbial quality of edible oyster (*Crassostrea madrasensis*). Durairaj *et al,* (1983) has reported his work on the bacterial quality of edible oyster from Tuticorin. His findings are more or less very similar to the work reported here. He found that TPC were in the range of 10^3-10^4 cfu/g in the oyster meat. He also did not detect any pathogen like *V.cholerae*, *V.parahaemolyticus* and *Salmonella.*

Colwell and Liston, (1960) has investigated the microbial profile of the Pacific oyster (*Crassostrea gigas*). They have found total plate count (TPC) of oyster meat in the range of 4 log cfu/ml. Motes *et al,* (1994) and Matte *et al,* (1994) have reported the presence of *V.cholerae* non-O1 in 21% of the Alabama Bay oyster (*Crassostrea gigas*) and 31% of oyster from Sao Paulo Brazil respectively. *Salmonella* was detected in 7.4% of the oyster harvested from the Atlanta coast of United States (Brands *et al,* 2005).

The farmed oysters of Kollam are totally pathogen free and the presence of Enterobacteriaceae and other coliform group of indicator bacteria can be substantially brought down by simple depuration process so that they become quite hygienic for export to the Western market.



4.1.8. Bacterial quality of dried fishes from the retail markets of Cochin

Fourty samples of commercially dried fish samples from the retail fish markets of Cochin were procured during the period 2004 and 2005 were studied for their bacterial quality. The samples consisted of 8 different species namely *Saurida* species (4 samples), *Leognathus* species (7 samples), *Otolithus* species (4 samples), *Scoliodon* species (5 samples), *Etroplus maculates* (5 samples), *Secutor* species (4 samples), *Anchovilla* species (6 samples) *Cynoglossus* species (5 samples). The bacterial quality including the presence of pathogens were investigated and the results are presented in Table 4.1.8. Total plate count (TPC) was more or less in the range of 10^3- 10^5 cfu/g. Coliforms including pathogens were very low being in the range of 0-25MPN. In four groups of the samples no coliform was detected. *E.coli* were not detected except two samples of *Cynoglossus*. Faecal streptococci were detected in a few samples in a very low range. However *S.aureus* were more or less present in all the species (10 samples) out of 40. *V.cholerae, V.parahaemolyticus* and *Salmonella* were not detected in any of the samples examined.

The data presented in Table 4.1.8 showed that generally the microbial quality of dried fishes were good, even though the presence of *S.aureus* was confirmed in at least 25% of samples analyzed in the range of 10^2-10^3 is indicative of unhygienic human handling.c

Basu *et al,* (1989) and Prasad *et al,* (1994) have made extensive studies on the bacterial quality of dried fishes from the inland markets of Kakinada (AndraPradesh). They have reported the total plate counts (TPC) in the range of 10^3 to 10^5 cfu/g and coliforms were in the range of 20-80/g. They could not detect any *E.coli*, faecal streptococci and coagulase positive *Staphylococcus aureus* in most of the samples examined. Lalitha and Surendran, (2002) has examined the bacterial quality of cured dried fishes from Cochin for *Clostridium botulinum* and has reported its presence. The data presented in table 4.1.8 are more or less in agreement with the findings of Basu *et al,* (1989) and Prasad *et al,* (1994).

4.1.8. Bacterial quality of dried fishes from the retail maekets of Cochin

Sl no	Sample	TPC	Total Coliforms	Faecal Coliforms	*E.coli*	Faecal Streptococci	*S.aureus*	*V.cholerae*	*V.para-haemolyticus*	*Salmonella*
1.	Pallikora (n= 4)	$4.2x10^5$cfu/g-$6.5x10^5$cfu/g	0-15	0-4.5	N.D	N.D	0-$4x10^3$cfu/g 2(+)	N.D	N.D	N.D
2.	Flat Mullan (n=7)	$4.8x10^4$cfu/g-$8x10^4$cfu/g	0-2.5	0-2.5	N.D	N.D	0-200 1(+)	N.D	N.D	N.D
3.	Kuttan (n=4)	$1.6x10^4$cfu/g-$3.26x10^5$cfu/g	0.4-25	0.4-4.5	N.D	N.D	N.D	N.D	N.D	N.D
4.	Shark (n=5)	$1.4x10^5$-$3.6x10^5$cfu/g	N.D	N.D	N.D	0-100	0-$3.5x10^3$ 2(+)	N.D	N.D	N.D
5.	Pearl spot (n=5)	$1.92x10^5$-$2.3x10^5$cfu/g	N.D	N.D	N.D	0-$2x10^3$	0-$3.3x10^3$ 3(+)	N.D	N.D	N.D
6.	Mullan (n =4)	$2.4x10^3$cfu/g-$3.8x10^4$cfu/g	N.D	N.D	N.D	N.D	0-$2x10^2$ 1(+)	N.D	N.D	N.D
7	Anchovy (n=6)	$1.1x10^3$cfu/g-$2.5x10^4$cfu/g	N.D	N.D	N.D	N.D	0-$3x10^2$ 1(+)	N.D	N.D	N.D
8.	Sole fish (n=5)	$1.71x10^4$cfu/g-$3.8x10^4$cfu/g	0-2.5	0-2.5	100 (2+)	10-80	N.D	N.D	N.D	N.D

4.1.9. Bacterial quality of dried shrimps from markets of Cochin

Twenty-four samples of marine shrimps belonging to *Metapenaeus dobsoni, M.affinis* and *Parapaenopsis stylifera* from the retail fish markets of Cochin were examined for their microbial quality including the presence of pathogens. The results are presented in Table 4.1.9.

The bacterial profiles of dried shrimps were similar to that of dried fishes in so far as the presence of *E.coli, V.cholerae, V.parahaemolyticus* and *Salmonella* are concerned. These pathogens were totally absent in the dried shrimps. Similarly total coliforms and faecal coliforms were also very minimal and the counts of faecal streptococci were also less as well as the presence of *S.aureus* was also limited and in positive samples, the *S.aureus* count did not exceed 10^2 cfu/g. However the total plate counts were very high mostly being in the range of 10^5-10^7cfu/g.

Valsan *et al,* (1985) reported the bacterial quality of dried non-penaeid fishes from the retail markets of Bombay. They reported a total bacterial count in the range of 10^3-10^6cfu/g. *E.coli* was absent in all the samples. *S.aureus* was detected in two samples in the range of 10^2/g. Faecal streptococci were detected only in two samples, that too in a very low count i.e. 30-3.5x10^2cfu/g. They reported the presence of *Salmonella enteritidis* in only one sample.

The low bacterial count in dried fishes and shrimps, compared with fresh fishes and shrimps can be attributed to the low water activity of these products, which will not support the growth of most of the bacteria. Most of the microbes including pathogenic bacteria grow most rapidly at levels of water activity in the range of 0.995- 0.980. (ICMSF1980). Troller and Stinson, (1975) reported that *S.aureus,* the most drought tolerant of the pathogenic bacteria has a lower limit variously reported on different substrates as between 0.86 – 0.83.

4.1.9. Bacterial quality of dried shrimps from the retail markets of Cochin

Sl. No.	Sample	TPC	Total Coliforms	Faecal Coliforms	*E.coli*	Faecal Streptococci	*S.aureus*	*V.cholerae*	*V.parahae-molyticus*	*Salmonella*
1	*M.dobsoni* (n=8)	$4.4x10^4$cfu/g-$3.6x10^7$cfu/g	2.5-4	0-2.5	N.D	N.D	0- $2x10^2$cfu/g	N.D	N.D	N.D
2	*M.affinis* (n=8)	$2.34x10^5$cfu/g-$3.6x10^7$cfu/g	2.5-4	0-2.5	N.D	0- $3x10^2$cfu/g	N.D	N.D	N.D	N.D
3	*P.stylifera* (n=8)	$1.33x10^6$cfu/g-$3.85x10^6$cfu/g	0-2.5	N.D	N.D	0-$3.8x10^3$cfu/g	0- $6x10^2$cfu/g	N.D	N.D	N.D

4.1.10. Bacterial quality of frozen shrimps from seafood factories and retail cold storages of Cochin

Block frozen and individually quick frozen shrimps from seafood processing factories and retail cold storages of Cochin were investigated for their bacterial quality as well as the presence of critical pathogens like *V.parahaemolyticus*, *V.cholerae* and *Salmonella*. A total of 17 frozen shrimp samples, 14 from seafood processing factories and 3 from local cold storages were included for the study. Four samples were IQF whole shrimps; others were block frozen peeled and undeveined (PUD) shrimps. Five of the samples were tiger prawns and 12 were white prawns (*P.indicus*). The results are presented in Table 4.1.10.

The total plate counts of the block frozen peeled and undeveined (PUD) shrimps from seafood processing factories were in the range of 10^3-10^5cfu/g while the samples from the local cold storages had total plate counts in 10^3 to 10^7/g. The individually quick frozen samples were procured only from factories and had total plate counts in the range of 10^4-10^6/g. For frozen shrimps, the EU countries and USFDA has fixed $5x10^5$/g as the maximum permissible limit of total plate count. On this basis, 12 samples out of the 17 samples had their total plate count in the acceptable limit.

The total coliforms and faecal coliforms were detected in 14 out of the 17 samples and 11 out of the 17 samples respectively, but only 2 of the samples had a total coliforms above 100/g. EU as well as USFDA and BIS do not stipulate any limit for total coliforms and faecal coliforms, but only the limit as 20/g as the upper acceptable limit for *E.coli*.

Ten out of 17 samples of frozen shrimp did not harbour any *E.coli*. Two samples had an *E.coli* count less than 20/g and 5 samples had their *E.coli* count above the approved limit of 20/g. Faecal streptococci were detected in the range of 10^2-10^3cfu/g in 9 samples. It is interesting to note that in all samples where *E.coli* was detected, faecal streptococci was also present. The *S.aureus* were detected in 9 out of the 17 samples in the range of 10^2-10^3/g. The upper limit of acceptability for *S.aureus* as per quality

4.1.10. Bacterial quality of frozen shrimp from seafood factories & cold storges of Cochin

Sl. No.	Sample*	TPC	Total Coliforms	Faecal Coliforms	*E.coli*	Faecal Streptococci	*S.aureus*	*V.cholerae*	*V.para-haemolyticus*	*Salmonella*
1	BFSPUD-T	$2.75x10^7$cfu/g	45	0.3	0.3	$4x10^2$cfu/g	$4x10^2$cfu/g	N.D	N.D	N.D
2	BFSPUD-W	$1.14x10^5$cfu/g	45	45	N.D	N.D	10^3	N.D	N.D	N.D
3	IQFS	$1.3x10^5$cfu/g	0.4	0.4	N.D	$2x10^2$cfu/g	N.D	N.D	N.D	N.D
4	BFSPUD-W (R)	$1.1x10^7$cfu/g	140	140	110	$2.74x10^4$	$4x10^3$cfu/g	Present	Present	N.D
5	BFSPUD-W (R)	$1.15x10^6$cfu/g	140	140	110	$2.2x10^3$cfu/g	10^3	Present	Present	Present
6	BFSPUD-W (R)	$1.69X10^6$cfu/g	45	45	25	$5x10^3$cfu/g	$2x10^3$cfu/g	Present	Present	N.D
7	BFSPUD-T	$6.4X10^4$cfu/g	45	45	N.D	N.D	100	Present	N.D	N.D
8	BFSPUD-T	$4.2x10^3$cfu/g	25	N.D	N.D	N.D	N.D	N.D	N.D	N.D
9	BFSPUD-T	$3.7x10^5$cfu/g	4.5	4.5	4.5	$3x10^2$cfu/g	$3x10^2$cfu/g	N.D	N.D	N.D
10	IQFS	$5.7x10^4$cfu/g	0.4	0.4	N.D	N.D	N.D	N.D	N.D	N.D
11	IQFS	$3.2x10^6$ cfu/g	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
12	FSPUD-W	$2.49x10^5$cfu/g	25	25	25	$6x10^2$cfu/g	$8x10^2$cfu/g	N.D	N.D	N.D
13	FSPUD-w	$6.64x10^5$cfu/g	4.5	4.5	100	$1.45x10^3$cfu/g	N.D	N.D	N.D	N.D
14	FSPUD-W	$5.6x10^4$cfu/g	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
15	FSPUD-T	$3.1x10^5$cfu/g	0.9	N.D	N.D	N.D	$6x10^2$ cfu/g	N.D	N.D	N.D
16	FSPUD-W	$1.53x10^5$cfu/g	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
17	IQFS	$1.2x10^5$cfu/g	0.4	N.D	N.D	$5x10^2$cfu/g	N.D	N.D	N.D	N.D

IQF- Individually quick frozen; BSFUD-T- Block frozen shrimp peeled and undeveined- tiger;BFSPUD-W-Block frozen shrimp peeled and undeveined –white, BFSPUD-w-Block frozen shrimp peeled and undeveined –white, (R)-Retail cold storages.

standards is 100/g, eight samples out of the 17 exceeded this limit. *V.cholerae* non-O1 was detected in 4 out of the 18 samples. Three of the samples were from local cold storages while one were from seafood factory. *V.parahaemolyticus* were detected from three samples of marine white shrimp and all the three samples were from retail cold storage. None of the samples from seafood processing plant carried *V.parahaemolyticus*. *Salmonella* was detected in only one sample and the samples had its origin from the retail cold storage.

A lot of studies have been reported on the microbial quality of frozen seafood meant for export. Varma *et al,* (1985) and (2003) have reported the bacterial quality of frozen processed shrimp from seafood factories in and around Cochin. They have found that 6.7-13% of the PD shrimp samples had a total plate count above the permitted limit of $5x10^5$ cfu/g. The *E.coli* count exceeded the limit of 20/g in 5.66% of the samples and *S.aureus* was detected in many samples but exceeded approved limit of 100/g only in one case. They detected *Salmonella* in two samples, *V.parahaemolyticus* in 10 samples and *V.cholerae* was detected in none of the samples. Iyer and Shrivastava, (1989c) has reported the incidence of *Salmonella* in 10% of the headless shell on frozen shrimps, 12% of PD shrimps and 14% of the PUD shrimps. Sanjeev *et al,* (2000) found that *V.parahaemolyticus* was present in 8 out of the 62 blocks frozen shrimp sample from industry.

The total bacterial count of individually quick frozen samples were in the range of 10^4-10^6cfu/g. *E.coli* was not detected in any of the samples. *S.aureus*, *V.cholerae*, *V.parahaemolyticus* and *Salmonella* were absent in all the samples of shrimp from industry analyzed. However a study carried out by Hatha *et al* (1998) have found the presence of *E.coli* in 2% of the total 1264 samples of raw IQF shrimps they have analyzed. They have also reported the presence of *S.aureus* in 1% and *Salmonella* in 0.1% of the samples analyzed. All the cooked IQF samples were free from these organisms.

4.1.11. Bacterial quality of frozen finfish from the retail markets of Cochin

A total of 24 samples of frozen finfish consisting of seer fish (*Scomberomorous species*, 6 sample), pearl spot (*Etroplus suratensis*, 5 samples), sea catla (*Lethrinus* species, 5 samples), tuna (*Thunnus species*, 5 samples) and pomfret (*Pampus argentius*, 3 samples) were studied for their bacterial quality including the presence of pathogenic bacteria. The results are presented in Table 4.1.11. The total bacterial count of the 24 samples of frozen finfish was in the range of 10^5-10^7 cfu/g. Most of the samples carried coliforms and *E.coli* ranging between 9.5-140^+. Faecal streptococci were in the range of 10^2-10^3cfu/g. *S.aureus* was detected in 3 species while seer fish and pomfret were free from *S.aureus*. *V.parahaemolyticus* was not detected in any of the samples while *V.cholerae* non-O1 and *Salmonella* was present in 2 samples each.

A few reports on the microbial quality of frozen finishes sold in the retail markets of Cochin and elsewhere are available. Nambiar and Surendran, (2003a) have made an elaborate study on the microbial quality of frozen finfish in the retail markets of Cochin. They have found that in 77% of samples analyzed the total plate count were above 10^6cfu/g. They reported that 6.3% of the samples examined carried the pathogen *Salmonella*. A similar investigation by Iyer and Shrivasthava, (1989c) have also reported that 25% of the frozen catfish samples and 20% of frozen seer fish samples in the retail market of Cochin carried *Salmonella*. Gnanambal and Pattterson, (2005) have studied the biochemical and microbiological quality of frozen finfishes of Tuticorin consisting of *Lethrinus* species, *Rastrelliger* species, *Stoleophorus* species, *Scombroides* species, *Scomberomorus* species and *Caranx* species. They found that the total plate counts (TPC) generally were in the range of 10^3/g and *E.coli* less than 100/g. They found the presence of *Salmonella* in some of the samples. Sanjeev *et al*, (2000) have found *Vibrio parahaemolyticus* in 9.42% of the frozen fish and shellfish species while Lakshmanan *et al*, (1991) found that generally the quality of the frozen fishes from the retail cold

4.1.11. Bacterial quality of frozen fin fishes from the retail markets of Cochin.

SL NO	Sample	TPC	Total Coliforms	Faecal Coliforms	Faecal Streptococci	*E.coli*	*S.aureus*	*V. cholerae*	*V. para-haemolyticus*	*Salmonella*
1	Seer fish (n=6)	$2.21x10^5$cfu/g - $1.89x10^5$cfu/g	25-110	25-110	$3x10^2$cfu/g- $2.2x10^3$cfu/g	0-110	N.D	N.D	N.D	N.D
2	Pearl spot (n=5)	$3.2x10^5$cfu/g- $4.7x10^6$cfu/g	0-140	140	0-$3.2x10^3$cfu/g	45-110	0-10^3cfu/g (5 +ve)	Present	N.D	Present
3	Catla (n=5)	$4.2x10^5$cfu/g- $3.58x10^7$cfu/g	45-95	45-95	0-$9.9x10^3$cfu/g	0-45	0- $3.3x10^3$cfu/g (3 +ve)	Present	N.D	N.D
4	Tuna (n=5)	$2x10^5$cfu/g- $1.14x10^6$cfu/g	0-140^+	110-140^+	0-$5.3x10^3$ cfu/g	110-140^+	0-$6x10^2$cfu/g (4 +ve)	N.D	N.D	N.D
5	Pomfret (n=3)	$2.21x10^5$ cfu/g- $4.03x10^5$cfu/g	9.5-45	9.5-45	0-$1.5x10^3$cfu/g	0-9.5	N.D	N.D	N.D	Present

storages were very good. They have reported very low counts for the total plate counts and also they could not detect coliforms, *E.coli*, Faecal streptococci, *S.aureus*, *V.cholerae*, *V.parahaemolyticus* and *Salmonella* in the samples analyzed.

The present study on the bacterial quality of frozen finfish from retail markets of Cochin indicated that generally the frozen finfishes available in the Cochin markets were of poor microbial quality. This observation is in full agreement with the findings of Nambiar and Surendran, (2003). The findings were in total disagreement with the results also obtained by Laksmanan *et al,* (1991).

4.1.12. Bacterial quality of value added fishery products from the retail markets of Cochin

A set of eighteen value added fishery products consisting of fish kheema (6 numbers), fish finger (2 numbers), fish steak (2 numbers), fish cutlet (5 numbers) and fish sausage (3 numbers) from the cold storages of Cochin were investigated for their bacterial quality including the presence of critical pathogens. The data are presented in Table 4.1.12. The total plate count of these value added fishery products, which are so processed that a final one step cooking treatment is required for consumption, were more or less in the range of 10^4cfu/g except a few samples of fish kheema (TPC in 10^6/g) and fish steak (TPC 10^5/g). The presences of coliforms were very low except one fish kheema sample, where the total coliforms were 140 MPN. The cases of faecal coliforms were similar to total coliforms but faecal streptococci and *E.coli* were not detected in any samples of fish steaks, fish cutlet and fish sausage. *V.cholerae*, *V.parahaemolyticus* and *Salmonella* were not detected in any of the eighteen samples tested; however *S.aureus* were invariably present in almost 66% of the value added fishery products.

From the standpoint of microbial quality parameters, most of the value added fishery products were in acceptable limit. Of these products, fish sausages were found to be better in microbial quality than others. But the presence of *S.aureus* in numbers exceeding 100/g in 66% of the 18 samples tested is a cause of concern from the food

4.1.12. Bacterial quality of value added fishery products from the retail markets of Cochin

Sl NO	Sample	TPC	Total coliforms	Faecal coliforms	*E.coli*	Faecal Streptococci	*Staph aureus*	*Vibrio Cholerae*	*Vibrio Para-haemolyticus*	*Salmonella*
1	Fish Kheema (n=6)	$4.9x10^4$cfu/g-$3.7x10^6$cfu/g	20-140	20-140	0-110	0-$4.5x10^4$cfu/g	0-200 (5 +ve)	N.D	N.D	N.D
2	Fish Finger (n=2)	$2x10^3$ - $2.35x10^5$cfu/g	0-25	0-25	N.D	N.D	0-10^2cfu/g (1+ve)	N.D	N.D	N.D
3	Fish Steaks (n=2)	$3x10^4$-$2.85x10^5$cfu/g	0-2.5	0-2.5	N.D	N.D	$3.2x10^2$-$4x10^3$cfu/g (2+ve)	N.D	N.D	N.D
4	Fish Cutlet (n=5)	$2.1x10^4$cfu/g-$4x10^4$cfu/g	0-2.5	0-0.4	N.D	N.D	$1.2x10^3$cfu/g-$3x10^2$cfu/g (2+ve)	N.D	N.D	N.D
5	Fish Sausage (n=3)	$7.4x10^4$cfu/g-$2x10^4$	N.D	N.D	N.D	N.D	0- $5x10^2$cfu/g (2+ve)	N.D	N.D	N.D

safety point of view. Most of the *S.aureus* could be potential producers of enterotoxins. Further it is well known that *S.aureus* originates from the food handlers, hence their presence in the value added fish products indicates the poor hygienic status of the food handlers.

The reports on the bacterial quality of value added fishery products are very scanty. Joseph *et al,* (1984) have studied the bacterial quality of fish cutlet from low priced fishes. They have followed the shelf life quality of the products by following changes in total plate count of the frozen cutlet. The total plate counts were in the range of 10^3/g in five days of frozen storage, it increased to 10^9/g within 12 days of frozen storage at -8°C. The product was free from coliforms, *E.coli*, faecal streptococci and coagulase positive *Staphylococcus aureus*. Baer *et al,* (1976) have made a detailed microbiological examination of the frozen breaded fish and shellfish in Washington, USA. They have studied the quality in terms of *E.coli*, faecal streptococci and *S.aureus* for breaded, precooked frozen samples of fish steaks, fish cake, crab cake, scallops, clam, heddoks, fish chips and shrimp. The total plate counts of these products were in the range of less than 100/g to 10^8/g with a geometrical mean of 450/g to $2.2x10^5$/g. coliforms (MPN) and *E.coli* (MPN) were also showed great fluctuations from sample to sample, being less than 2 to $1.1x10^5$cfu/g for total coliforms and less than 3 to $2.1x10^4$/g for *E.coli* respectively. *S.aureus* were detected in all the samples ranging from less than 3 to 2400/g.

Surkiewicz *et al,* (1968) have made a bacteriological survey of the frozen prepared food industry and the forth scientific study report presented the bacterial quality of of frozen breaded fish from Washington D.C. He had found a very high total plate counts for the products from 18 processing plants. The MPN coliforms of the sample varied between less than 3 to 1100/g while the presence of *E.coli* and *S.aureus* were comparatively less. Much other works from the value added products from seafood industry have not been recorded. The present report appear to be a pioneering work so far

as a detailed study has been made from the bacterial quality of value added products from India.

4.2. *Staphylococcus aureus* in fish and fishery products

4.2.1. Incidence of coagulase positive *S.aureus* in fish and fishery products

So far, the data on bacterial quality of fresh fish and shellfish, frozen fish and shellfish and dried fish and shellfish including value added fishery products have been presented and discussed (Table 4.1.1. to 4.1.12). In these data, the quality parameters like total plate count, total coliforms, faecal coliforms, faecal streptococci and *S.aureus* as well as the critical pathogens like *V.cholerae*, *V.parahaemolyticus* and *Salmonella* have been detailed. Even though pathogens like *V.cholerae*, *V.parahaemolyticus* and *Salmonella* are found in relatively low percentages in the samples studied, their presence were not in a very alarming level. Further this critical pathogens were either part of fish and shellfish (eg: *V.parahaemolyticus*) or the pathogens have their source, the process water or the polluted water from were they were harvested; but the significant observation made in this study was the invariable presence of *S.aureus* in a sizable proportion of fish and fishery products investigated.

Table 4.2.1.gives the incidence of *S.aureus* in the 257 fish and fishery products analyzed. Except green mussel, fresh clams and farmed oyster samples, all other eleven out of fourteen groups of fish and fishery products were found to be contaminated with *S.aureus* in varying levels. The presence of *S.aureus* was the lowest of 8.3% in dried shrimp samples, 11% in fresh shrimp from retail trade, 20-25% in samples of fresh crab, finfish, boiled clam meat and dried fishes. Their incidence was 52% in frozen shrimp and 50% each in frozen finfishes and fresh cephalopods. The incidence was 66.66% in value added fishery products. In total 25.29% of the 257 seafood samples analyzed carried *S.aureus*. This is a matter of concern because of the fact that (1) *S.aureus* strains are

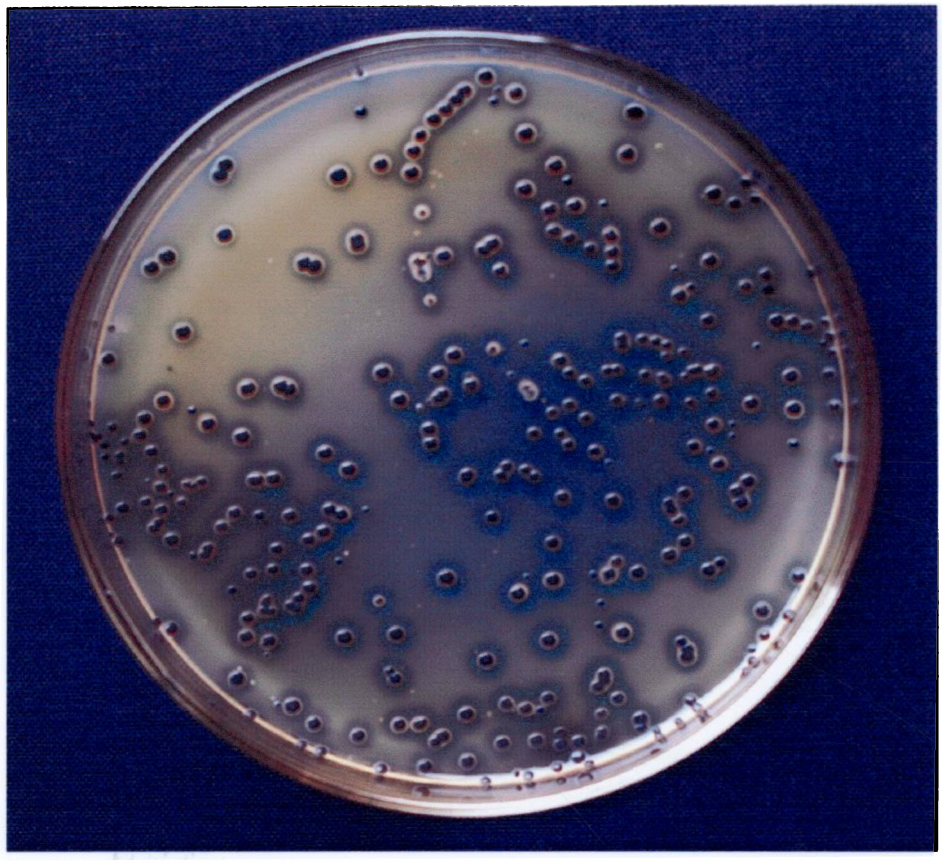


Fig.1. *Staphylococcus* colonies on Baird-Parker agar

2.1. Incidence of S.aureus in fish and fishery products studied

Sl.No	Fish/fish products	No of samples analyzed	No of samples positive for *S.aureus*	% of incidence
1	Fresh fin fish	51	11	21.4%
2	Fresh shrimp	18	2	11.1%
3	Fresh crab	10	2	20%
4	Fresh cephalopods	8	4	50%
5	Fresh green mussel	18	N.D	N.D
6	Fresh clam	11	N.D	N.D
7	Boiled clam meat	9	2	22%
8	Oyster	9	N.D	N.D
9	Dried fishes	40	10	25%
10	Dried shrimps	24	2	8.3%
11	Frozen shrimp	17	8	52.94%
12	Frozen fin fish	24	12	50%
13	Value added fishery products	18	12	66.66%
14	All the samples (Total)	257	65	25.29%

potential source of thermostable enterotoxins and (2) they are mainly sourced from unhygienic fish handlers.

Gram-negative pathogens are very susceptible to heat treatment and are destroyed at temperature between 60 to 70°C. So they cannot be considered as very dangerous in the case of raw seafood products which will be cooked before consumption, however the presence of *S.aureus* in raw food as well as value added products has to be viewed with concern because under ambient conditions of storage, they could produce thermostable enterotoxins which will survive even 121°C of heat processing. So on the standpoint of food safety, *S.aureus* is a more critical pathogen than the Gram-negative pathogens; hence a detailed investigation has been made on the incidence, toxigenicity, growth kinetics and biochemical characteristics of *S.aureus* strains isolated from fish and fishery products.

As stated in Table 4.2.1, 25.29% of the fish and fishery products investigated have been found to carry *S.aureus*. Many reports on the incidence of *S.aureus* in freshfish/shellfish and various fishery products have been published. Sanjeev *et al,* (1985) have investigated 50 samples of cooked, picked and frozen crabmeat from the local markets of Cochin. They found a very high incidence (68%) of *S.aureus* in the samples tested with a population range of 10^3-10^5 cfu/g. Sanjeev *et al* 1986 and 1996 have made further studies on the incidence of *S.aureus* in fish and fishery products. They have isolated 89 *S.aureus* strains from these products. Lakshmanan *et al,* (1984), Iyer *et al,* (1986), Jeyasekharan and Ayyappan, (2002), Antony *et al,* (2004), Prasad *et al,* (1994) and Desai and Kamat, (1998) have also reported the incidence of *S.aureus* in fresh, frozen and dried fish and fishery products from India. Silverman *et al,* (1961) and Sumner *et al,* (1982) have also isolated *S.aureus* from uncooked shrimp samples and peeled and deveined cooked shrimp samples. The presences of *S.aureus* in fresh fish and shellfish from Netherlands have been reported by Vanden Broke *et al,* (1984).

4.2.2. Biochemical characteristics of coagulase positve *S.aureus* of fish and fishery products

Of the 150 isolates of *Staphylococcus* cultures giving typical reaction on Baird-Parker agar, ie, black 2-4 diameter colonies with a white margin and a zone of clearance around the colony, only fifty strains gave typical coagulase reaction in the tube test using rabbit plasma EDTA reagent (Difco, USA). This means only one third of the isolates were typical *S.aureus* cultures and the remaining were coagulase negative *Staphylococcus* even though they gave standard reactions on Baird-Parker.

Of the 50 coagulase positive *Staphylococcus* only 25 were selected for further study viz; detailed cultural, biochemical and toxicological investigations. Critical biochemical characteristics of the 25 cultures are presented in Table 4.2.2. The Table gives the characteristics like Gram reaction, pigment production, catalase reaction, utilization of glucose(H&L oxidative/ fermetative metabolism), coagulase reaction, urease production and thermo nuclease activity. All the cultures gave typical Gram positive staining and were cocci, arranged in pairs or bunch. Thirteen cultures out of 25 produced golden yellow pigment at ambient temperature (28±2°C) temperature as well as 37°C while 6 cultures produced pale yellow pigments. Six cultures did not produce any pigment in these two temperatures. All the cultures were catalase positive and produced acid without gas from glucose. All were positive for coagulase reaction as well as urease production. Twenty-four out of 25 cultures showed thermonuclease activity at 37°C while one strain was negative for thermo nuclease activity.

Bergy's manual on systematic bacteriology vol.2 (Sneath *et al*, 1986) describes the characteristics of *S.aureus*. According to these descriptions 90% or more of the strains are positive for pigmentation and produces catalase and has respiratory as well as fermentative metabolism in the case of glucose. Nearly all strains produce coagulase enzyme and thermonuclease. Hence all 25 strains included in the study show characteristic typical reactions as per Bergy's manual of systematic bacteriology.

4.2.2. Biochemical characteristics of *S. aureus* from fish and fishery products

ure No	Gram reaction	Pigment production	Catalase reaction	H&L reaction glucose (fermentation with out gas)	Coagulase Reaction	Urease reaction	Thermonuclease activity
G1	+ve cocci	Golden yellow	+ve	+ve	+ve	+ve	+ve
G2	+ve cocci	Golden yellow	+ve	+ve	+ve	+ve	+ve
G3	+ve cocci	Golden yellow	+ve	+ve	+ve	+ve	+ve
G4	+ve cocci	Pale Yellow	+ve	+ve	+ve	+ve	+ve
G5	+ve cocci	Golden yellow	+ve	+ve	+ve	+ve	+ve
G6	+ve cocci	Golden yellow	+ve	+ve	+ve	+ve	+ve
G7	+ve cocci	Golden yellow	+ve	+ve	+ve	+ve	+ve
G8	+ve cocci	Pale yellow	+ve	+ve	+ve	+ve	+ve
G9	+ve cocci	No pigment	+ve	+ve	+ve	+ve	+ve
G10	+ve cocci	No pigment	+ve	+ve	+ve	+ve	+ve
G11	+ve cocci	Pale yellow	+ve	+ve	+ve	+ve	+ve
G12	+ve cocci	Pale yellow	+ve	+ve	+ve	+ve	+ve
G13	+ve cocci	Yellow	+ve	+ve	+ve	+ve	+ve
G14	+ve cocci	Golden yellow	+ve	+ve	+ve	+ve	+ve
G15	+ve cocci	Golden yellow	+ve	+ve	+ve	+ve	+ve
G16	+ve cocci	Golden yellow	+ve	+ve	+ve	+ve	+ve
G17	+ve cocci	No Pigment	+ve	+ve	+ve	+ve	+ve
G18	+ve cocci	No pigment	+ve	+ve	+ve	+ve	+ve
G19	+ve cocci	Pale yellow	+ve	+ve	+ve	+ve	+ve
G20	+ve cocci	Golden yellow	+ve	+ve	+ve	+ve	+ve
G21	+vecocci	Golden yellow	+ve	+ve	+ve	+ve	+ve
G22	+ve cocci	Golden yellow	+ve	+ve	+ve	+ve	+ve
G23	+ve cocci	No pigments	+ve	+ve	+ve	+ve	+ve
G24	+ve cocci	No pigment	+ve	+ve	+ve	+ve	-ve
G25	+ve cocci	Golden yellow	+ve	+ve	+ve	+ve	+ve

4.2.3. Time taken for coagulase reaction and virulence potential of *S.aureus* from fish and fishery products

The coagulase reactions of the 25 *S.aureus* strains were determined by the tube test method (Difco manual, 1998) because tube test are reported as more reliable. The Table 4.2.3 shows the time taken, for a positive coagulase reaction at 37°C in a serological water bath. Based on the time taken the virulence potential was rated as 4^+ if the time taken is 30 minute or less, 3^+ if the time taken is between 31 and 60 minutes and so on. The twenty-one out of 25 (84%) of the *S.aureus* cultures tested gave a positive coagulase reaction in less than 30 minutes indicating their very high virulence potential. The remaining 4 cultures (16%) gave a positive reaction within 31-60 minutes showing a 3^+ virulence potential rating.

According to Difco manual (1998) any degree of clotting of the rabbit plasma with EDTA reagent in 3-4 hours is considered as a positive coagulase reaction. Coagulase reaction is generally considered, as an indication of virulence potential of *S.aureus* cultures and strains, which are of significance in infection or food poisoning, usually will clot within 4 hours of incubation with the reagent at 37°C. Many weak enzyme producing strains will coagulate the plasma only after over night incubation.

Eighty four per cent of strains in the present study clotted rabbit plasma with EDTA within 30 minutes and remaining 16% in the next 60 minutes. Sperber and Tatini, (1975) recommended that a 4^+ reaction should stand alone for the definitive identification of *S.aureus*. According to AOAC protocols (AOAC, 1990) every result ($1^+$to 4^+) are considered as positive while APHA (Lancette and Tatini, 1992) considers only 3^+ and 4^+ as positive results.

Coagulase and thermonuclease are produced by most of the strains of *S.aureus*. There appears to be no significant difference in pattern of production between enterotoxigenic and non-enterotoxigenic strains of *S.aureus* (Varnam and Evans, 1991).

4.2.3. Coagulase reaction time and virulence potential of *S.aureus* cultures from fish and fishery products

Time taken for positive reaction	Virulence rating	No of cultures positive	% of the total
30 minutes or less	++++	21	84%
31 to 60 minutes	+++	4	16%
61 to 90 minutes	++	-	-
120 minutes- 4 hours	+	-	-
Above 4 hours	-	-	-

4.2.4. Extracellular virulence enzymes associated with *S.aureus* from fish and fishery products

S.aureus strains have the potential to produce several exoenzymes that contribute to their virulence (Dinges *et al,* 2000). These enzymes are lypolysins, proteolysins, haemolysins, phosphatases, and thermo nuclease etc. In the case of the 25 *S.aureus* strains from fish and fishery products, a detailed investigation have made on these extracellular virulence enzymes and results are presented in following sub-sections.

4.2.4.1. Lipolytic enzymes of *S.aureus* strains from fish and fishery products on tributyrine agar

The lipolytic enzyme activity of the 25 *S.aureus* strains was determined on tributyrin agar. Tributyrin was incorporated in standard nutrient agar plates as described in the materials and methods section (Chapter 3). The lipolytic activity was determined by measuring the diameter of zone of clearance after 24 hours incubation of the plates at 37°C. In order to calculate the reactive potential of the cultures for the purpose of comparison of activity between cultures, the diameter of the zone of clearence was divided by the colony diameter and ratios obtained was considered as lypolytic activity index of each cultures. The results are presented in Table 4.2.4.1.

All the 25 *S.aureus* strains tested displayed lipolytic activity against tributyrin. The lipolytic zone diameter varied between 13 mm and 17mm and the corresponding lipolytic activities varied between 1.44 and 2.125. The maximum lipolytic activity index of 2 - 2.125 was exhibited by eleven *S.aureus* strains, medium lipolytic activity in the range of 1.672 - 1.88 was shown by 13 strains and the least lipolytic index of 1.44 was showen by only one strain.

4.2.4.2. Lecithinase activity (phospholipase activity) of *S.aureus* from fish and fishery products

Phospholipase activities of *S.aureus* strains were determined on egg yolk agar. The lecithinase activity zone diameter was calculated after 48 hours incubation of the test

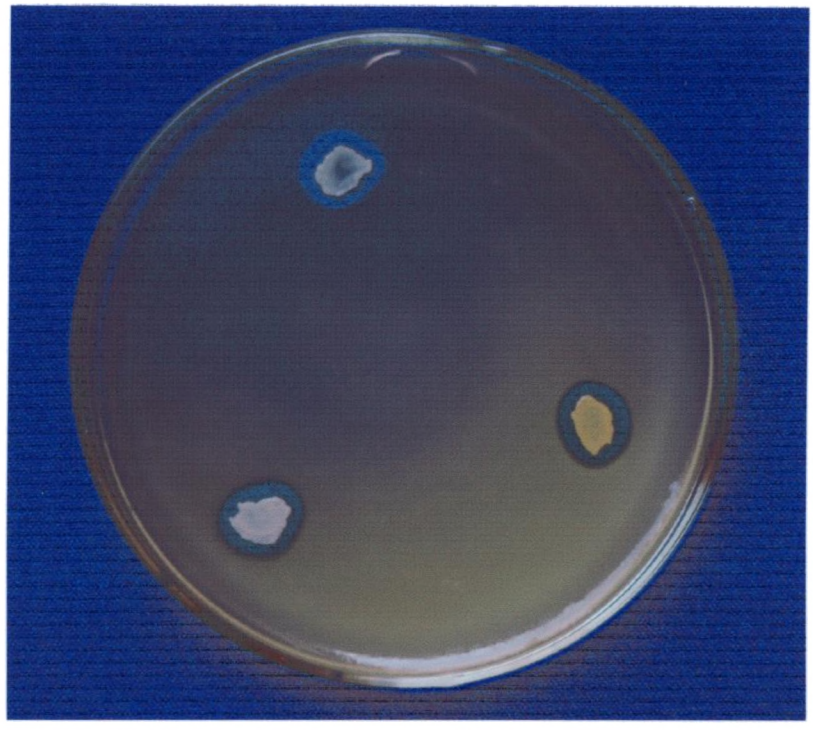


Fig.2. Lipolytic activity of *Staphylococcus* on tributyrin agar

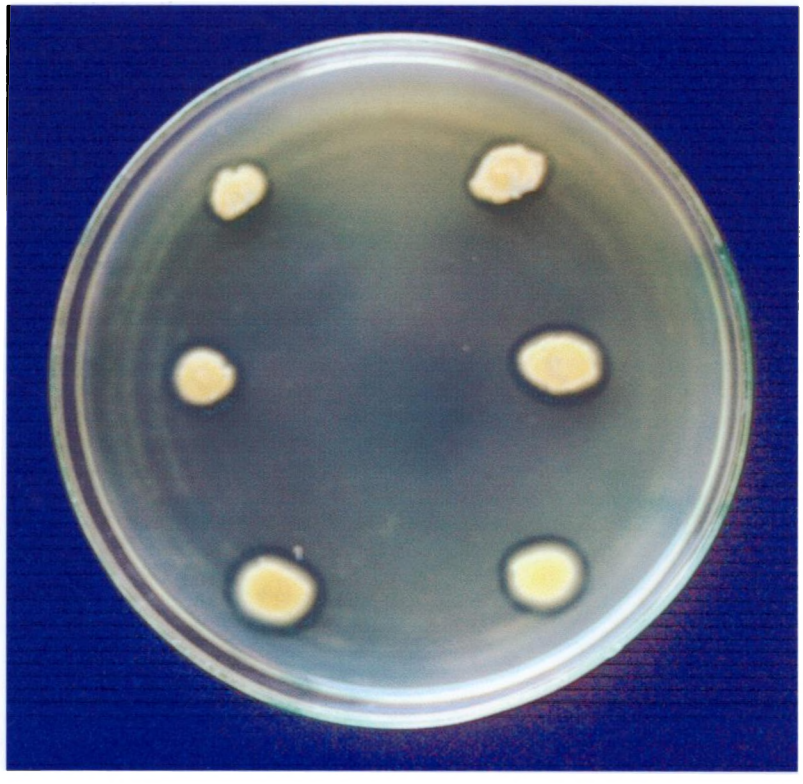


Fig.3. Lecithinase activity of *Staphylococcus* on egg yolk agar

4.2.4.1. Lipolytic enzyme activity of *S. aureus* strains on Tributyrin agar

Culture No	Colony diameter	Lipolysis zone diameter	Lipolytic activity index*
CG1	8 mm	14 mm	1.75
CG2	8 mm	17 mm	2.125
CG3	8 mm	16 mm	2
CG4	8 mm	15 mm	1.875
CG5	8 mm	15 mm	1.67
CG6	9 mm	15 mm	1.67
CG7	8 mm	16 mm	2
CG8	8 mm	15 mm	1.67
CG9	9 mm	17 mm	1.88
CG10	8 mm	17 mm	2.125
CG11	8 mm	17 mm	2.125
CG12	8 mm	16 mm	2
CG13	8 mm	14 mm	1.75
CG14	8 mm	14 mm	1.75
CG15	8 mm	14 mm	1.75
CG16	8 mm	17 mm	2.125
CG17	8 mm	16 mm	2
CG18	8 mm	15 mm	1.67
CG19	8 mm	15 mm	1.67
CG20	8 mm	17 mm	2.125
CG21	8 mm	16 mm	2
CG22	8 mm	15 mm	1.67
CG23	8 mm	16 mm	2
CG24	9 mm	13 mm	1.44
CG25	9 mm	16 mm	1.77

Lipolytic activity index = $\frac{\text{Lipolysis zone diameter}}{\text{Colony diameter}}$

4.2.4.2. Lecithinase activity of *S. aureus strains* on egg yolk agar

Culture No	Colony diameter	Lecithinase activity zone diameter	Lecithinase activity index
CG1	9 mm	18 mm	2
CG2	9 mm	20 mm	2.22
CG3	10 mm	19 mm	1.9
CG4	9 mm	19 mm	2.11
CG5	9 mm	18 mm	2
CG6	10 mm	15 mm	1.5
CG7	8 mm	17 mm	2.125
CG8	9 mm	18 mm	2
CG9	8 mm	19 mm	2.37
CG10	10 mm	20 mm	2
CG11	8 mm	17 mm	2.125
CG12	9 mm	19 mm	2.11
CG13	9 mm	19 mm	2.11
CG14	10 mm	19 mm	1.9
CG15	9 mm	17 mm	1.89
CG16	8 mm	18 mm	2.25
CG17	8 mm	17 mm	2.125
CG18	10 mm	19 mm	1.9
CG19	10 mm	21 mm	2.12
CG20	9 mm	20 mm	2.22
CG21	9 mm	19 mm	2.11
CG22	10 mm	21 mm	2.1
CG23	9 mm	13 mm	1.44
CG24	9 mm	12 mm	1.33
CG25	9 mm	13 mm	1.44

Lecithinase activity index = $\frac{\text{Lecithinase activity zone diameter}}{\text{Colony diameter}}$

plates at 37°C and lecithinase activity index was calculated as in the case of lipolytic activity index by dividing the lecithinase activity zone diameter by the colony diameter of each culture. The results are presented in Table 4.2.4.2.

The lecithinase activity zone diameters of the 25 *S.aureus* strains were in the range of 12-21mm in diameter. The maximum zone size of 20-21 mm diameter was exhibited by 5 *S.aureus* strains while the medium zone diameter of 15-19 mm was given by 17 strains and the lowest zone diameter group of 12-13 mm was given by only three *S.aureus* strains. The lecithinase activity index of 2 and above was exhibited by 17 strains of *S.aureus* while 4 strains gave lecithinase activity index of about 1.9 and 4 strains gave a lecithinase activity index of about 1.5 or less.

Both lipase and phospholipase have attracted great attention from microbiologists as seen from publications (Nygran *et al*, 1966; Baird-Parker, 1969). Lipases are a group of enzymes that contributes to the virulence of *S.aureus*. *S.aureus* produces lipases in infected patients; further more lipase interferes with the phagocytosis of the infectious lipases producing *S.aureus* cells by host granulocytes thus indicating a direct involvement of lipases in pathogenesis (Mosbah *et al*, 2005; O'leary and Weld, 1964).

Lipase initiates the spreading of cutaneous and subcutaneous tissue through phophatidyl-inositol specific phosphlipase C activity (Sandel and Mc Killip, 2004).

The presences of lipases have been considered as an important character in the initiation of boils and carbuncles in humans and majority of *S.aureus* strains isolated from human infection posesses this enzyme (Milgrom and Flanagan, 1982).

Bhat *et al,* (1990) correlated pigment production with lipase production. They reported that golden colored isolates of *S.aureus* only produced these lipolytic enzymes. But no such correlation could be found in the present study. Irrespective of pigment production, all isolates golden coloured pigment producing, yellow pigment producing as

well as producing no pigment at all exhibited both lipase activities namely lipolytic activity against tributyrin and lecithine (egg yolk lipid).

4.2.4.3. Proteolytic enzyme activity of *S.aureus* strains isolated from fish and fishery products

Proteolytic enzyme activity of the 25 strains of *S.aureus* isolated from fish and fish products were determined on two separate media, namely casein agar as well as gelatin agar. The proteolytic enzyme activities are expressed as proteolytic zone diameter and relative proteolytic activity index was calculated as the ratio of proteolytic zone diameter divided by colony diameter of the culture in the respective media. The results are presented in Table 4.2.4.3.1 and 4.2.4.3.2.

All the 25 cultures exhibited very good proteolytic enzyme activity on casein agar. The proteolysis zone diameter varied from 15mm to 22mm. most of the strains produced proteolytic zones of diameter above 20mm. Only 3 strains produced zones below 20mm diameter. Accordingly the proteolytic activity indices were also very high for almost all the cultures.

On the contrary, proteolytic activity of *S.aureus* strains was very little on gelatin agar. Out of 25 strains tested only 5 could produce proteolysis on gelatin agar the rest of the cultures even though showed good growth on culture medium did not show proteolytic activity. So it is concluded that while proteases capable of hydrolyzing casein were universal in the *S.aureus* strains, the presence of gelatinase enzyme was very limited. The Bergy's manual of systematic bacteriology (vol.2 Sneath *et al* 1986) states that most strains of *S.aureus* will hydrolyze native animal proteins like egg white, casein and haemoglobin, as well as polypeptide like gelatin. In the present study, it is proved that *S.aureus* strains universally produced proteolytic enzyme activity against casein, but only 5 out of the 25(20%) strains produced gelatinolytic enzymes.

Martley *et al,* (1970) typed coagulase positive Staphylococci in to different groups on the basis of proteolytic activity, on buffered casein agar. They could not find any

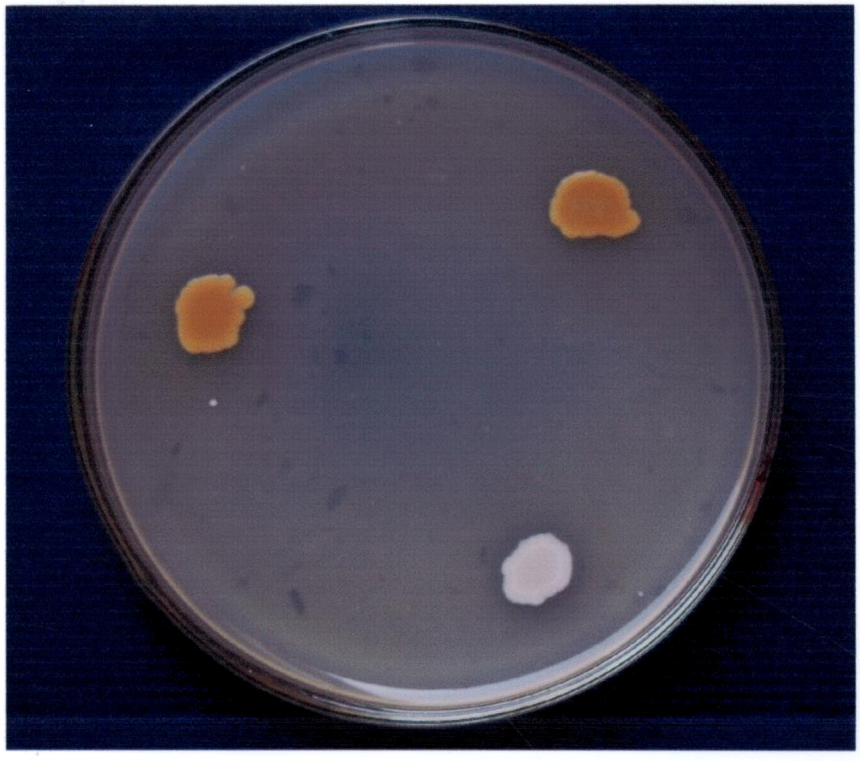


Fig.4. Proteolytic activity of *Staphylococcus* on casein agar

4.2.4.3.1. Proteolytic enzyme activity of *S. aureus* strains on casein agar

Culture No	Colony diameter	Proteolysis zone diameter	Proteolytic activity index
CG1	9 mm	22 mm	2.44
CG2	10 mm	21 mm	2.1
CG3	9 mm	22 mm	2.44
CG4	9 mm	22 mm	2.4
CG5	10 mm	24 mm	2.4
CG6	11 mm	15 mm	1.36
CG7	10 mm	19 mm	1.9
CG8	9 mm	24 mm	2.67
CG9	9 mm	22 mm	2.4
CG10	10 mm	20 mm	2
CG11	10 mm	24 mm	2.4
CG12	10 mm	21 mm	2.1
CG13	10 mm	23 mm	2.3
CG14	10 mm	18 mm	1.8
CG15	10 mm	20 mm	2
CG16	8 mm	22 mm	2.75
CG17	9 mm	22 mm	2.44
CG18	10 mm	22 mm	2.2
CG19	10 mm	21 mm	2.1
CG20	10 mm	22 mm	2.2
CG21	9 mm	22 mm	2.4
CG22	9 mm	22 mm	2.4
CG23	10 mm	22 mm	2.2
CG24	10 mm	21 mm	2.1
CG25	9 mm	17 mm	1.88

Proteolytic activity index = $\frac{\text{Proteolysis zone diameter}}{\text{Colony diameter}}$

4.2.4.3.2. Proteolytic activity of *S. aureus* strains on gelatin agar

Culture No	Colony diameter	Proteolysis zone diameter	Proteolytic index
CG1	9 mm	-	-
CG2	9 mm	17 mm	1.9
CG3	10 mm	18 mm	1.8
CG4	9 mm	-	-
CG5	9 mm	-	-
CG6	10 mm	-	-
CG7	8 mm	-	-
CG8	9 mm	-	-
CG9	10 mm	18 mm	1.8
CG10	10 mm	17 mm	1.7
CG11	8 mm	-	-
CG12	9 mm	-	-
CG13	9 mm	19 mm	2.1
CG14	10 mm	-	-
CG15	9 mm	-	-
CG16	9 mm	-	-
CG17	8 mm	-	-
CG18	10 mm	-	-
CG19	10 mm	-	-
CG20	9 mm	-	-
CG21	9 mm	-	-
CG22	10 mm	-	-
CG23	9 mm	-	-
CG24	9 mm	-	-
CG25	9 mm	-	-

correlation of proteolytic activity with any of the tested parameters like phage typing, enterotoxin production or source of strains. Bhat *et al,* (1990) has reported that all clinical isolates of *S.aureus* produced gelatinase enzymes. But Clark *et al,* (1961) found that *S.aureus* cultures isolated from milk did not liquefy gelatin. The findings in this study that majority of *S.aureus* isolates from fish and fishery products are not gelatinolytic, is in full agreement to the observations of Clark *et al,* (1961).

4.2.4.4. Phosphatase and thermonuclease activities of *S.aureus* strains isolated from fish and fishery products

Phosphatase activity of the 25 strains of *S.aureus* was tested on phenolphthalein diphosphate agar. The thermonuclease activity was tested on DNase test agar as described in chapter 3. The data are presented in Table 4.2.4.4. Also the data on coagulase activity of strains are also combined in this Table for comparison.

It can be seen from the Table that all the 25 strains produced phosphatase. Only 24 out of the 25 were found to produce thermo nuclease activity. The deviation observed in the case of thermonuclease activity of one strain was rechecked by repeated experiments, but the negative reaction was confirmed.

Thermo nuclease is a heat stable endo or exo nuclease capable of hydrolyzing DNA and RNA in host cells (Sandel and McKillip, 2004). According to Bergy's manual (Sneath *et al,* 1986) more than 90% of the *S.aureus* strains are positive for coagulase, phosphatase and thermo nuclease test. Da Silva *et al,* (2000) have also reported that 97.8% of *S.aureus* strains they studied produced thermo nuclease. Bennet *et al,* (1986) found that 93% of the *S.aureus* strains isolated from foods and food ingredients were DNAse positive. Chang and Huang, (1995) found 95% of the *S.aureus* strains they studied were capable of producing thermonuclease . The present study also is in full agreement with these reports.

All the *S.aureus* isolates in the present study showed phosphatase activity. Bhat *et al* (1990) have also made similar observations. Langlois *et al* (1990) had reported that

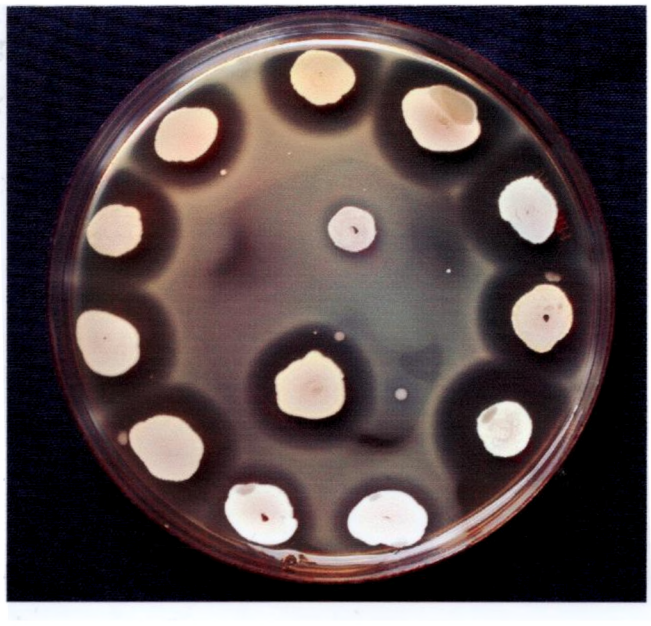


Fig. 5 Thermo nuclease activity of *Staphylococcus* on DNAse test agar

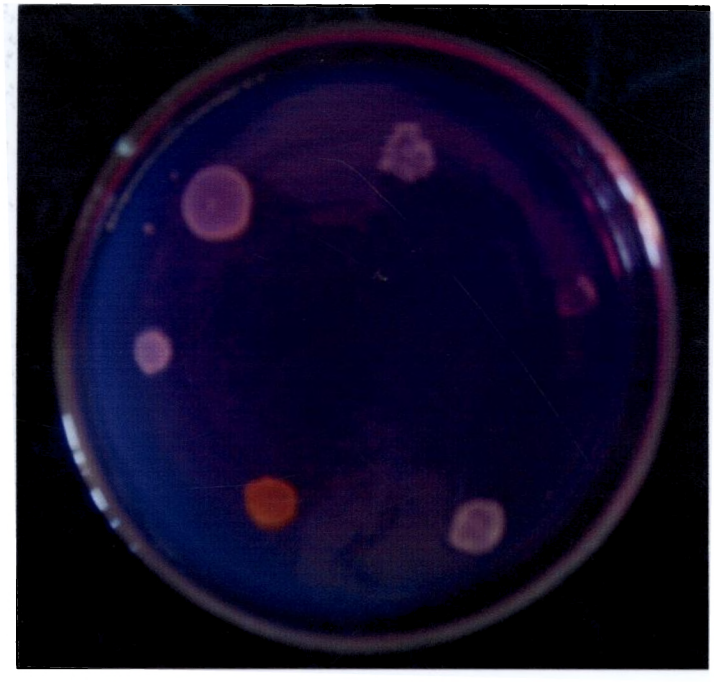


Fig.6. Phosphatase activity of *Staphylococcus* on Phenolphthalein di phosphate agar

4.2.4.4. Thermonuclease and Phosphatase activities of *S.aureus* strains *vis-à-vis* their coagulase reaction

Culture No	Coagulase test	Thermonuclease activity	Phosphatase activity
CG1	+ve	+ve	+ve
CG2	+ve	+ve	+ve
CG3	+ve	+ve	+ve
CG4	+ve	+ve	+ve
CG5	+ve	+ve	+ve
CG6	+ve	+ve	+ve
CG7	+ve	+ve	+ve
CG8	+ve	+ve	+ve
CG9	+ve	+ve	+ve
CG10	+ve	+ve	+ve
CG11	+ve	+ve	+ve
CG12	+ve	+ve	+ve
CG13	+ve	+ve	+ve
CG14	+ve	+ve	+ve
CG15	+ve	+ve	+ve
CG16	+ve	+ve	+ve
CG17	+ve	+ve	+ve
CG18	+ve	+ve	+ve
CG19	+ve	+ve	+ve
CG20	+ve	+ve	+ve
CG21	+ve	+ve	+ve
CG22	+ve	+ve	+ve
CG23	+ve	+ve	+ve
CG24	+ve	-ve	+ve
CG25	+ve	+ve	+ve

96.5% of *S.aureus* strains of human origin produced phosphatase. They have also found that phosphatase activity was also exhibited by coagulase negative *Staphylococcus*. The present study showed 100% correlation between phosphatase activity and coagualse reaction.

4.2.4.5. Haemolytic activity of *S.aureus* from fish and fishery products

The haemolytic activity (Haemolysin production) of 25 *S.aureus* strains from fish and fishery products were determined on blood agar(human blood 5% level). Data are presented in Table 4.2.4.5. Of the 25 cultures tested, 2 were α-haemolytic (partial haemolysis), 15 were β- haemolytic (complete haemolysis) and 9 were γ-haemolytic(non-haemolytic). Of the 2 α-haemolytic strains, one exhibited β- haemolytic activity on incubation up to 48 hours while the 2nd one remained α-haemolytic even after 48-hours of incubation.

Decomposition of blood (haemolysis) is generally considered as a critical reaction indicative of pathogenesis as well as toxigenesis by bacteria. At least four different haemolysins are reported, namely α, β, γ and δ haemolysins. β- haemolytic activity is considered as an important virulence property of the pathogen. According to Bergy's manual (vol.2, Sneath *et al*, 1986) nearly all strains of *S.aureus* produce one or a combination of all this haemolysins. β- haemolysin is produced frequently by strains of animal origin. It is a phospholipase C.

Staphylococcal haemolysins are identified as important virulence factors that contribute to bacterial inversion and escape from host immune response (Salyers and Whitt, 1994). Jarvis and Lawrence, (1971) reported the production of haemolysins by both enterotoxigenic and non-enterotoxigenic strains of *S.aureus*. Meyer, (1967) and Dimitracopoulos *et al,* (1976) reported that β- haemolysin production is indicative of *S.aureus* of animal origin. Dimitracopoulos and Papavasilliou, (1971), Samaraki *et al,* (1969) found that *S.aureus* producing β- haemolysin alone or in combination with α and β

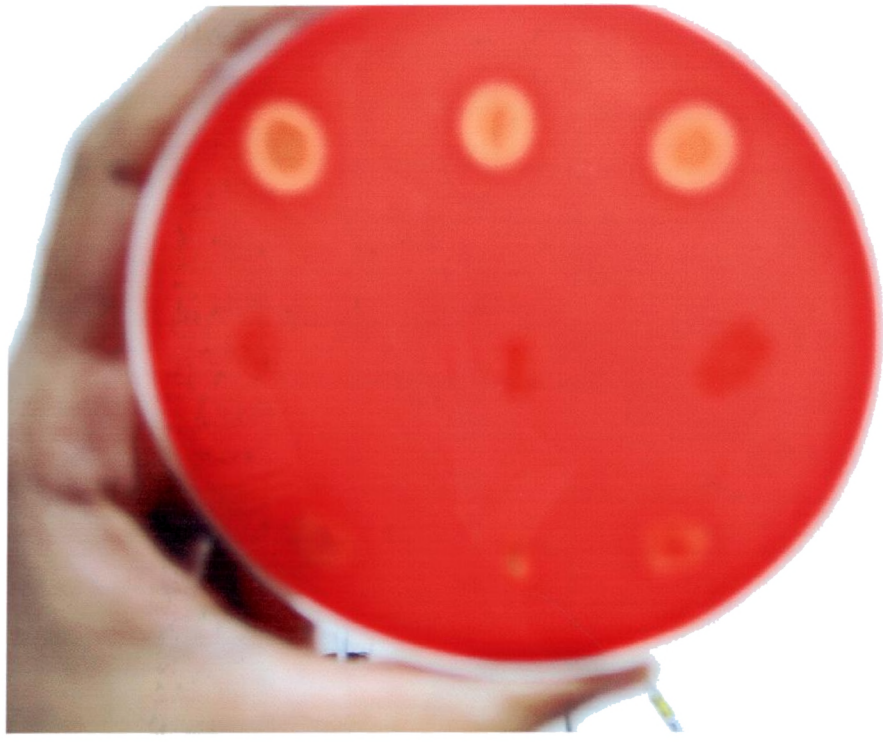


Fig.7. Haemolytic activity of *Staphylococcus* on blood agar

4.2.4.5. Haemolytic activity of *S. aureus* strains from fish and fishery products

Culture No	Haemolytic activity (Blood agar)	
	α	β
CG1	-ve	-ve
CG2	+ve	+ve
CG3	-ve	+ve
CG4	+ve	-ve
CG5	-ve	-ve
CG6	-ve	+ve
CG7	-ve	+ve
CG8	-ve	+ve
CG9	-ve	+ve
CG10	-ve	+ve
CG11	-ve	-ve
CG12	-ve	+ve
CG13	-ve	+ve
CG14	-ve	-ve
CG15	-ve	-ve
CG16	-ve	-ve
CG17	-ve	+ve
CG18	-ve	+ve
CG19	-ve	+ve
CG20	-ve	+ve
CG21	-ve	+ve
CG22	-ve	+ve
CG23	-ve	-ve
CG24	-ve	-ve
CG25	-ve	-ve

haemolysis are peculiar to nasal areas of healthy human beings. In the present study 60% of the 25 *S.aureus* strains tested were β-haemolytic and this could indicate that they might have originated from the nasal areas of fish handlers.

4.2.5. Antibiotic sensitivity pattern of *S.aureus* from fish and fishery products

Antibiotics employed for studying the sensitivity of *S.aureus* strains towards antibiotics listed in section 3.1.5 in the Materials and Methods section. Twelve antibiotics namely Chloramphenicol, Tetracycline, Oxolinic acid, Oxytetracycline, Nalidixic acid, Neomycin, Sulphamethaxazole, Erythromycin, Vancomycin, Novobiocin, Methicillin and Penicillin were tested against these cultures and sensitivity was measured as the diameter of zone of clearance obtained. It can be seen from the table that all the isolates have developed resistance towards Nalidixic acid (100%). The resistance towards other antibiotics were Oxolinic acid (72%) Penicillin (56%), Neomycin (12%), Tetracycline (12%), Oxytetracycline (12%), Erythromycin (8%), Sulphamethaxazole (4%) and Chloramphenicol (4%) but none of the antibiotics showed resistance to Vancomycin, Novobiocin and Methicillin.

The study carried out by Sanjeev and Iyer (1988) in *S.aureus* isolates from fish processing factory workers showed that about 64.75% of the isolates were resistant to Ampicillin followed by penicillin (59.84%). Resistance towards other antibiotics was in the order:- Tetracycline (22.95%), Erythromycin (7.38%), Neomycin (5.74%), Chloramphenicol (3.28%). Sanjeev *et al* (1985) found that *S.aureus* isolates from frozen fishery products showed highest percentage of resistance to Ampicillin (76.92%) followed by Penicillin (76%) and Tetracycline (34.62%).

Comparing with the results of the above studies, the antibiotic resistance in the current study is more. The high incidence of antibiotic resistant *S.aureus* isolates in fish and fish products indicated the prevalence of antibiotic resistant strains in handlers since this organism is considered as an indicator of personal hygiene. The study carried out by

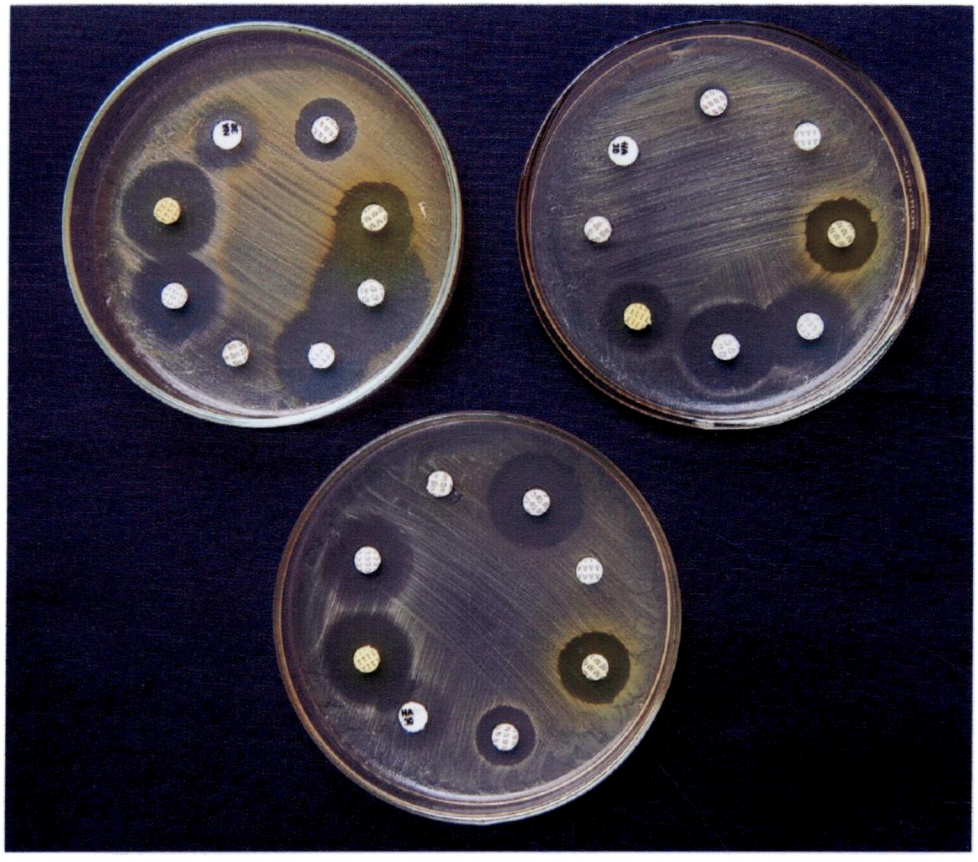


Fig. 8. Antibiotic sensitivity of *Staphylococcus* on Muller Hinton Agar

4.2.5. Antibiotic sensitivity pattern of *S. aureus* cultures from fish and fishery products

Antibiotic disc used	Sensitive	%	Intermediate	%	Resistant	%
Chloramphenicol	24	96%	-	-	1	4%
Neomycin	21	84%	1	4%	3	12%
Tetracycline	8	32%	14	56%	3	12%
Oxytetracycline	13	52%	9	36%	3	12%
Nalidixic acid	-	-	-	-	25	100%
Sulphamethaxazole	24	96%	-	-	1	4%
Oxolinic acid	7	28%	-	-	18	72%
Novobiocin	25	100%	-	-	-	-
Vancomycin	25	100%	-	-	-	-
Methicillin	25	100%	-	-	-	-
Erythromycin	14`	56%	9	36%	2	8%
Penicillin	11	44%	-	-	14	56%

Bhat *et al* (1991) confirmed that human isolates of *S.aureus* were more resistant to penicillin.

S.aureus is frequently resistant to antibiotic therapy due to its capacity to produce an exopolysaccharide barrier and because of its location within the micro abscesses, which limit the action of drugs (Jeljaszewicz *et al*, 2000). Even though most of the strains in the present study are susceptible to most of the antibiotics like Chloramphenicol, Nalidixic acid, Novobiocin, Vancomycin, Methicillin and Erythromycin, attention should be given to the fact that the *S.aureus* strains have demonstrated resistance to one or more antibiotics.

In the present study 56% of the *S.aureus* strains tested were resistant to Penicillin. This result is not surprising because penicillin is one of the most commonly used antibiotic for the treatment of infections in humans and animals. Different rate of penicillin resistance have been reported for *S.aureus* obtained from different sources. Aarestrup *et al,* (2000) reported that only 7% of *S.aureus* cultures isolated from infections of poultry were resistant to penicillin. Acco *et al*, (2003) showed that 70% of the *S.aureus* strains isolated from food handlers were resistant to penicillin.

4.2.6. Carbohydrate utilization by *S.aureus* strains isolated from fish and fishery products

The carbohydrate utilization of *S.aureus* strains isolated from fish and fishery products was determined against a set of pentoses, hexoses, disaccharides and sugar derivatives in order to ascertain whether there is any bio grouping possible based on sugar metabolism.

4.2.6.1. Utilization of pentoses

Three pentoses namely arabinose, ribose and xylose were studied for their acid production by *S.aureus* in broth culture and the data are presented in Table 4.2.6.1. All the 25 *S.aureus* cultures fermented ribose with the production of acid but no gas, but none

4.2.6.1.Utilisation of Pentoses by *S.aureus* cultures

Culture No	L-Arabinose	D-Ribose	D-Xylose
CG1	-ve	+ve	-ve
CG2	-ve	+ve	-ve
CG3	-ve	+ve	-ve
CG4	-ve	+ve	-ve
CG5	-ve	+ve	-ve
CG6	-ve	+ve	-ve
CG7	-ve	+ve	-ve
CG8	-ve	+ve	-ve
CG9	-ve	+ve	-ve
CG10	-ve	+ve	-ve
CG11	-ve	+ve	-ve
CG12	-ve	+ve	-ve
CG13	-ve	+ve	-ve
CG14	-ve	+ve	-ve
CG15	-ve	+ve	-ve
CG16	-ve	+ve	-ve
CG17	-ve	+ve	-ve
CG18	-ve	+ve	-ve
CG19	-ve	+ve	-ve
CG20	-ve	+ve	-ve
CG21	-ve	+ve	-ve
CG22	-ve	+ve	-ve
CG23	-ve	+ve	-ve
CG24	-ve	+ve	-ve
CG25	-ve	+ve	-ve

of the cultures metabolized arabinose and xylose. According to Bergy's manual of Systematic bacteriology (vol.2, Sneath *et al*, 1986), above 90% of the *S.aureus* cultures utilized D-ribose while less than 90% of the cultures could metabolize L-arabinose and D-xylose. Langlois *et al,* (1990) reported that among the three pentoses, D-xylose and L-arabinose were not utilized by any of the *S.aureus* cultures they studied while 91.4% of their *S.aureus* cultures utilized D-ribose. The results obtained in the present study are in agreement with these results.

4.2.6.2. Utilization of hexoses

Utilization of five hexoses including D-mannitol by the 25 *S.aureus* strains from fish and fishery products were studied and the results are presented in Table 4.2.6.2. It was found that all the 5 hexoses were utilized by all the 25 *S.aureus* strains with the production of acid without gas. According to Bergy's manual of systematic bacteriology (vol.2, Sneath *et al,* 1986). *S.aureus* produce acid from all these sugars both aerobically and anaerobically. Findings in this study are in full agreement with the Bergy,s manual. Further studies of Langlois *et al,* (1990) have showed that the *S.aureus* isolates of human origin fermented galactose (89.5%), fructose (100%) mannose (100%) and mannitol (96.5%), while the results in the case of *S.aureus* isolates of bovine origin were positive for acid production from galactose (89.6%), fructose (100%), mannose (100%) and mannitol (93.2%).

4.2.6.3. Utilization of disaccharides by *S.aureus* cultures from fish and fishery products

The results of utilization studies of disaccharides by 25 cultures are presented in Table 4.2.6.3. All the 25 cultures utilized lactose, maltose, sucrose and trehalose with the production of acid but none of the cultures could utilize D-cellobiose. Bergy's manual of systematic bacteriology has recorded that more than 90% of *S.aureus* cultures are positive for utilization of lactose and maltose. Langlois *et al,* (1990) had also reported 84.2% of

4.2.6.2. Utilisation of hexoses by *S.aureus* isolated from fish and fishery products

Culture No	D-Fructose	D-Galactose	D-Glucose	D-Mannitol	D-Mannose
CG1	+ve	+ve	+ve	+ve	+ve
CG2	+ve	+ve	+ve	+ve	+ve
CG3	+ve	+ve	+ve	+ve	+ve
CG4	+ve	+ve	+ve	+ve	+ve
CG5	+ve	+ve	+ve	+ve	+ve
CG6	+ve	+ve	+ve	+ve	+ve
CG7	+ve	+ve	+ve	+ve	+ve
CG8	+ve	+ve	+ve	+ve	+ve
CG9	+ve	+ve	+ve	+ve	+ve
CG10	+ve	+ve	+ve	+ve	+ve
CG11	+ve	+ve	+ve	+ve	+ve
CG12	+ve	+ve	+ve	+ve	+ve
CG13	+ve	+ve	+ve	+ve	+ve
CG14	+ve	+ve	+ve	+ve	+ve
CG15	+ve	+ve	+ve	+ve	+ve
CG16	+ve	+ve	+ve	+ve	+ve
CG17	+ve	+ve	+ve	+ve	+ve
CG18	+ve	+ve	+ve	+ve	+ve
CG19	+ve	+ve	+ve	+ve	+ve
CG20	+ve	+ve	+ve	+ve	+ve
CG21	+ve	+ve	+ve	+ve	+ve
CG22	+ve	+ve	+ve	+ve	+ve
CG23	+ve	+ve	+ve	+ve	+ve
CG24	+ve	+ve	+ve	+ve	+ve
CG25	+ve	+ve	+ve	+ve	+ve

4.2.6.3. Utilisation of disaccharides by *S.aureus* cultures from fish and fishery products

Culture No	D-Cellobiose	Lactose	Maltose	Sucrose	D-Trehalose
CG1	-ve	+ve	+ve	+ve	+ve
CG2	-ve	+ve	+ve	+ve	+ve
CG3	-ve	+ve	+ve	+ve	+ve
CG4	-ve	+ve	+ve	+ve	+ve
CG5	-ve	+ve	+ve	+ve	+ve
CG6	-ve	+ve	+ve	+ve	+ve
CG7	-ve	+ve	+ve	+ve	+ve
CG8	-ve	+ve	+ve	+ve	+ve
CG9	-ve	+ve	+ve	+ve	+ve
CG10	-ve	+ve	+ve	+ve	+ve
CG11	-ve	+ve	+ve	+ve	+ve
CG12	-ve	+ve	+ve	+ve	+ve
CG13	-ve	+ve	+ve	+ve	+ve
CG14	-ve	+ve	+ve	+ve	+ve
CG15	-ve	+ve	+ve	+ve	+ve
CG16	-ve	+ve	+ve	+ve	+ve
CG17	-ve	+ve	+ve	+ve	+ve
CG18	-ve	+ve	+ve	+ve	+ve
CG19	-ve	+ve	+ve	+ve	+ve
CG20	-ve	+ve	+ve	+ve	+ve
CG21	-ve	+ve	+ve	+ve	+ve
CG22	-ve	+ve	+ve	+ve	+ve
CG23	-ve	+ve	+ve	+ve	+ve
CG24	-ve	+ve	+ve	+ve	+ve
CG25	-ve	+ve	+ve	+ve	+ve

human isolates utilized lactose and 100% each maltose, sucrose and trehalose. They have also found that 100% of *S.aureus* isolates of bovine origin could utilize lactose and trehalose and 98.9% maltose. The data presented in Table 4.2.6.3 are in full agreement with the findings of Langlois *et al,* (1990) and the records in Bergy's manual of Systematic bacteriology (vol.2, Sneath *et al*,1986).

4.2.6.4. Utilization of trisaccharides and sugar derivatives by *S.aureus* cultures from fish and fishery products

Utilization of two trisaccharides, namely raffinose and melezitose and one glucose derivative, salicin (hydroxy methyl phenyl glucose) were also included in the study. The results are presented in Table 4.2.6.4. None of the 25 strains of *S.aureus* tested could utilize the trisaccharides as well as salicin. Baird-Parker, (1963; 1965), Kloos and Schleifer, (1975) have reported that *S.aureus* were not capable of utilizing the trisaccharides, raffinose and melezitose. The Bergy's manual of Systematic Bacteriology (vol.2, Sneath *et al,* 1986) has recorded that *S.aureus* are not able to metabolize raffinose, D-melezitose as well as salicin. The results obtained in this study are in full agreement with these reports. However there is a report from Langlois *et al,* (1990) who have recorded that *S.aureus* isolates of human origin and 13.6% isolates of bovine origin utilized raffinose and melezitose. But none of their cultures could utilize salicin, as found in this study.

4.2.6.5. Aminoacid utilization of coagulase positive *S.aureus* strains isolated from fish and fishery products

Utilization of four aminoacids namely arginine, histidine, lysine and ornithine by *S.aureus* cultures were determined in the aminoacid decarboxylase broth as described in the materials and methods section.The results are presented in Table 4.2.6.5.

All the 25 *S.aureus* cultures metabolized arginine but none of the cultures could metabolise histidine, lysine and ornithine. Krasuski, (1981) has found that ammonia was produced from arginine, but lysine and ornithine are not decarboxylated by *S.aureus*

4.2.6.4. Utilisation of other sugars and sugar derivatives by *S.aureus* cultures from fish and fishery products

Culture No	D-Melezitose	Raffinose	Salicin
CG1	-ve	-ve	-ve
CG2	-ve	-ve	-ve
CG3	-ve	-ve	-ve
CG4	-ve	-ve	-ve
CG5	-ve	-ve	-ve
CG6	-ve	-ve	-ve
CG7	-ve	-ve	-ve
CG8	-ve	-ve	-ve
CG9	-ve	-ve	-ve
CG10	-ve	-ve	-ve
CG11	-ve	-ve	-ve
CG12	-ve	-ve	-ve
CG13	-ve	-ve	-ve
CG14	-ve	-ve	-ve
CG15	-ve	-ve	-ve
CG16	-ve	-ve	-ve
CG17	-ve	-ve	-ve
CG18	-ve	-ve	-ve
CG19	-ve	-ve	-ve
CG20	-ve	-ve	-ve
CG21	-ve	-ve	-ve
CG22	-ve	-ve	-ve
CG23	-ve	-ve	-ve
CG24	-ve	-ve	-ve
CG25	-ve	-ve	-ve

4.2.6.5. Amino acid utilization by *S.aureus* strains isolated from fish and fishery products

Culture No	Amino acids used			
	L-Arginine	L-Histidine	L-Lysine	L-Ornithine
CG1	+ve	-ve	-ve	-ve
CG2	+ve	-ve	-ve	-ve
CG3	+ve	-ve	-ve	-ve
CG4	+ve	-ve	-ve	-ve
CG5	+ve	-ve	-ve	-ve
CG6	+ve	-ve	-ve	-ve
CG7	+ve	-ve	-ve	-ve
CG8	+ve	-ve	-ve	-ve
CG9	+ve	-ve	-ve	-ve
CG10	+ve	-ve	-ve	-ve
CG11	+ve	-ve	-ve	-ve
CG12	+ve	-ve	-ve	-ve
CG13	+ve	-ve	-ve	-ve
CG14	+ve	-ve	-ve	-ve
CG15	+ve	-ve	-ve	-ve
CG16	+ve	-ve	-ve	-ve
CG17	+ve	-ve	-ve	-ve
CG18	+ve	-ve	-ve	-ve
CG19	+ve	-ve	-ve	-ve
CG20	+ve	-ve	-ve	-ve
CG21	+ve	-ve	-ve	-ve
CG22	+ve	-ve	-ve	-ve
CG23	+ve	-ve	-ve	-ve
CG24	+ve	-ve	-ve	-ve
CG25	+ve	-ve	-ve	-ve

strains. According to Bergy's manual of systematic bacteriology (vol.2, Sneath *et al*, 1986) while *S.aureus* generally hydrolyzed arginine, reaction could be occasionally weak. But no work on the utilization of histidine by *S.aureus* could be found in the literature. The present report on utilization of amino acid by *S.aureus* is in general agreement with the recorded literature.

4.2.7. Biochemical and cultural characterization of coagulase negative *Staphylococcus* cultures from fish and fishery products.

Out of the 150 *Staphylococcus* colonies giving typical characteristic reactions on Baird-Parker agar, nearly 100 were found to be coagulase negative. Of these 25 purified typical cultures were selected for detailed biochemical and cultural characterization. The data are presented in Tables 4.2.7 and 4.2.7 (a).

Table 4.2.7 gives details of their Gram reaction, H&L O/F reaction for utilization of glucose, coagulase test, urease test and thermonuclease test, carbohydrate fermentation, haemolysin production etc. These characteristics are grouped into Tables for the purpose of their biological classification as per Harvey and Gilmour, (1985).

All the cultures were typically Gram +ve cocci, as in the case of *S.aureus*. Except the culture CG12 which produced a yellow pigment, other cultures produced either white or cream white pigment (no pigmentation). All of them were catalase positive, could ferment glucose with acid and decomposed urea. None of them produced coagulase or thermonuclease enzyme. All these reactions were common for all the 25-coagulase negative cultures but they showed different reactions in other bio chemical test as shown in Table 4.2.7(a).

Based on the above characteristics the coagulase negative staphylococci were classified into four species. Twenty-three out of the twenty-five isolates were classified in to four species two cultures were untypable. Seven of the cultures were identified as *S.epidermidis*, seven as *S.hysicus chromogens*, seven cultures *S.simu*lans and two as *S.warneri*.

4.2.7. Biochemical characteristics of coagulase negative *Staphylococcus* cultures from fish and fishery products

ture	Gram reaction	Pigment production	Catalase reaction	H&L reaction glucose (acid no gas)	Coagulase test	Urease Test	Thermonuclease Test
G 1	+ve cocci	White	+ve	+ve	-ve	+ve	-ve
G 2	+ve cocci	white	+ve	+ve	-ve	+ve	-ve
G 3	+ve cocci	Cream	+ve	+ve	-ve	+ve	-ve
G 4	+ve cocci	White	+ve	+ve	-ve	+ve	-ve
G 5	+ve cocci	Cream	+ve	+ve	-ve	+ve	-ve
G 6	+ve cocci	Cream	+ve	+ve	-ve	+ve	-ve
G 7	+ve cocci	White	+ve	+ve	-ve	+ve	-ve
G 8	+ve cocci	White	+ve	+ve	-ve	+ve	-ve
G 9	+ve cocci	White	+ve	+ve	-ve	+ve	-ve
G 10	+ve cocci	White	+ve	+ve	-ve	+ve	-ve
G 11	+ve cocci	White	+ve	+ve	-ve	+ve	-ve
G 12	+ve cocci	Yellow	+ve	+ve	-ve	+ve	-ve
G 13	+ve cocci	Cream	+ve	+ve	-ve	+ve	-ve
G 14	+ve cocci	White	+ve	+ve	-ve	+ve	-ve
G 15	+ve cocci	White	+ve	+ve	-ve	+ve	-ve
G 16	+ve cocci	White	+ve	+ve	-ve	+ve	-ve
G 17	+ve cocci	Cream	+ve	+ve	-ve	+ve	-ve
G 18	+ve cocci	Cream	+ve	+ve	-ve	+ve	-ve
G 19	+ve cocci	Cream	+ve	+ve	-ve	+ve	-ve
G 20	+ve cocci	White	+ve	+ve	-ve	+ve	-ve
G 21	+ve cocci	White	+ve	+ve	-ve	+ve	-ve
G 22	+ve cocci	White	+ve	+ve	-ve	+ve	-ve
G 23	+ve cocci	White	+ve	+ve	-ve	+ve	-ve
G 24	+ve cocci	white	+ve	+ve	-ve	+ve	-ve
G 25	+ve cocci	white	+ve	+ve	-ve	+ve	-ve

4.2.7(a). Classification of coagulase negative Staphylococci (Harvey and Gilmour 1985)

Coagulase	Thermo nuclease	Haemolysis	Acetoin	Pigment	Sucrose	Trehalose	Mannitol	Cellobiose	Maltose	Mannose	Xylose	Phosphatase	Novobiocin	Species identified
-ve	-ve	+ve	+ve	-ve	+	+	+	-	+	+	-	-	S	*S.simulans*
-ve	-ve	-ve	+ve	-ve	+	-	-	-	+	+	-	+	S	*S.epidermidis*
-ve	-ve	-ve	+	+	+	+	+	-	+	+	-	+	S	*S.hysicus sub sp.chromogens*
-ve	-ve	+	+	-ve	+	+	+	-	+w	+	-	-	S	*S.simulans*
-ve	-ve	-	+	+ve	+	-	+	-	+w	+	-	+	S	*S.hysicus sub sp.chromogens*
-ve	-ve	-	+	+	+	+	+	-	+w	+	-	-	S	*S.simulans*
-ve	-ve	-ve	+	-ve	+	-	+	-	+w	+	-	+	S	*S.hysicus sub sp.chromogens*
-ve	-ve	-ve	+	-ve	+	+	+	-	+w	+	-	+	S	*S.hysicus sub sp.chromogens*
-ve	-ve	+	+	-ve	+	+	+	-	+w	+	-	-	S	*S.simulans*
-ve	-ve	+	+	-ve	+	+	+	-	+w	+	-	-	S	*S.simulans*
-ve	-ve	+	+	-ve	+	+	+	-	+w	+	-	-	S	*S.simulans*
-ve	-ve	-ve	+	+ve	+	+	+	-	+w	+	-	+	S	*S.hysicus sub sp.chromogens*
-ve	-ve	-ve	+	+ve	+	+	+	-	+w	+	-	+	S	*S.hysicus sub sp.chromogens*
-ve	-ve	+	+	-	+	-	+	-	+w	+	-	-	S	*Untypable*
-ve	-ve	+	+	-	+	-	+	-	+w	-	-	+	S	*S.simulans*
-ve	-ve	-	+	-ve	+	+	+	-	+w	+	-	-	S	*S.warneri*
-ve	-ve	-	+	+	+	-	-	-	+d	+	-	+	S	*S.hysicus sub sp.chromogens*
-ve	-ve	-ve	+	+ve	+	+	+	-	+w	+	-	+	S	*S.warneri*
-ve	-ve	-	+	+ve	+	+	+	-	+w	+	-	+	S	*Untypable*
-ve	-ve	-	+w	+ve	-	-	+	-	+w	+		+	+	*S.epidermidis*
-ve	-ve	+	+	-	+	-	-	-	+w	+	-	+	S	*S.epidermidis*
-ve	-ve	-	+	-	+	-	-	-	+w	+	-	+	S	*S.epidermidis*
-ve	-ve	+	+	-	+	-	-	-	+w	+	-	+	S	*S.epidermidis*
-ve	-ve	-	+	-	+	-	-	-	+w	+	-	+	S	*S.epidermidis*
-ve	-ve	+	+	-	+	-	-	-	+w	+	-	+	S	*S.epidermidis*

The result of classification of coagulase negative cultures from fish and fishery products in to species is probably the first such report from India. The Bergy's manual of Systematic Bacteriology (vol.2, Sneath *et al*, 1986) has reported 19 species of *Staphylococcus*. All the four species we have identified are also included in these 19 species. For classifying the isolates in this study, the scheme of Harvey and Gilmour, (1985) was used because of the simplicity of the classification scheme and it was found that such classification well fitted to the Bergy's scheme as well. Of the four species of coagulase negative staphylococci isolated from seafood, *S.epidermidis*, *S.simulans* and *S.warneri* are usually found on skin and naturally we can conclude that they have reached the seafood from human handlers. However *S.hysicus sub species chromogens* occurs commonly on the skin of pig and less frequently on the skin of cattle and poultry (Bergy's manual of Systematic bacteriology, Vol .2. Sneath *et al,* 1986).

4.2.8. Extracellular virulence factors associated with coagulase negative *Staphylococcus* cultures isolated from fish and fishery products

Just like coagulase positive *S.aureus*, coagulase negative *Staphylococcus* cultures are also known to produce extracellular virulence factors like lipases, proteolytic enzymes, phosphatases and haemolysins. The potential to produce these virulence factors by the 25-coagulase negative cultures were studied and results are presented in 4.2.9.

4.2.9 Extracellular enzymes

4.2.9.1. Lipolytic activity of coagulase negative staphylococci against tributyrine

Lipolytic activity of coagulase negative *Staphylococcus* cultures against tributyrin is shown in Table 4.2.9.1. All 25 cultures produced lipolysis with zone diameter varying between 10-18mm. The lipolytic activity indexes calculated by dividing the lipolytic zone diameter with the colony diameter of the cultures are also shown in the Table. Maximum lipolytic activity index of two and above was exhibited by eight cultures, medium lipolytic activity as indicated by lipolytic activity index of 1.62 to 2 was given by 9

4.2.9.1. Lipolytic activity of coagulase negative *Staphylococcus* cultures on Tributyrine agar

Culture No	Colony diameter	Lipolysis zone diameter	Lipolytic activity index
NCG1	8 mm	16	2
NCG2	8 mm	14	1.75
NCG3	8 mm	13	1.63
NCG4	8 mm	15	1.9
NCG5	8 mm	18	2.25
NCG6	9 mm	10	1.11
NCG7	8 mm	12	1.5
NCG8	8 mm	11	1.37
NCG9	9 mm	14	1.55
NCG10	8 mm	18	2.25
NCG11	8 mm	18	2.25
NCG12	8 mm	14	1.75
NCG13	8 mm	16	2
NCG14	8 mm	14	1.75
NCG15	8 mm	16	2
NCG16	8 mm	18	2.25
NCG17	8 mm	14	1.75
NCG18	8 mm	10	1.25
NCG19	8 mm	11	1.37
NCG20	8 mm	16	2
NCG21	8 mm	10	1.25
NCG22	8 mm	14	1.75
NCG23	8 mm	15	1.87
NCG24	9 mm	14	1.55
NCG25	9 mm	15	1.67

cultures and the remaining 8 cultures shown a low lipolytic activity index between 1.25-1.55.

Bhat *et al,* (1990) have studied the lipolytic activity of fourty coagualase negative *Staphylococcus* cultures of human origin and found that 17.5% of the cultures were lipolytic against tributyrin. Troller and Bozman (1970) also reported the production of lipases by coagulase negative *Staphylococcus* isolates from human skin. In the present study all the 25 cultures tested exhibited lipolytic activity against tributyrin.

4.2.9.2. Lecithinase activity of coagulase negatve *Staphylococcus* from fish and fishery product

The lecithinase activity of coagulase negative cultures was determined on egg yolk agar and the lecithinase activity index was calculated by dividing the zone of clearence with the colony diameter of the culture. The data is presented in Table 4.2.9.2. It can be seen that all the 25 cultures tested showed lecithinase activity on egg yolk agar. For the isolation of *Staphylococcus* on Baird-Parker agar, the lecithinase activity of the cultures on egg yolk is a critical reaction. The phospholipo- protein lipase produced by *Staphylococcus* is responsible for the lecithinase activity. The enzyme activity is common for coagulase positive as well as coagulase negative *Staphylococcus* (Bergy's manual of Systematic bacteriology, vol.2. Sneath *et al*, 1986).

4.2.9.3. Proteolytic activity of coagulase negative *Staphylococcus* cultures

Proteolytic activity of 25 coagulase negative *Staphylococcus* cultures isolated from fish and fishery products were determined on casein and gelatin agar and the data are presented in Table 4.2.9.3.1. and 4.2.9.3.2.

On casein agar, all the 25 coagulase negative *Staphylococcus* cultures exhibited very good proteolytic activity, while on gelatin agar only 5 out of the 25 (20%) of the cultures could demonstrate proteolytic activity. It is interesting to note that similar

4.2.9.2. Lecithinase activity of coagulase negative *Staphylococcus* cultures on egg yolk agar

Culture No	Colony diameter	Lecithinase activity zone diameter	Lecithinase activity index
NCG1	9 mm	13	1.44
NCG2	9 mm	15	1.66
NCG3	10 mm	15	1.5
NCG4	9 mm	14	1.55
NCG5	9 mm	13	1.44
NCG6	10 mm	14	1.44
NCG7	8 mm	15	1.87
NCG8	9 mm	14	1.55
NCG9	8 mm	13	1.62
NCG10	10 mm	14	1.4
NCG11	8 mm	15	1.8
NCG12	9 mm	13	1.44
NCG13	9 mm	12	1.33
NCG14	10 mm	14	1.4
NCG15	9 mm	13	1.44
NCG16	8 mm	12	1.5
NCG17	8 mm	14	1.75
NCG18	10 mm	12	1.2
NCG19	10 mm	13	1.3
NCG20	9 mm	14	1.55
NCG21	9 mm	14	1.55
NCG22	10 mm	13	1.3
NCG23	9 mm	12	1.33
NCG24	9 mm	14	1.55
NCG25	9 mm	15	1.66

4.2.9.3.1. Proteolytic activity of coagulase negative *Staphylococcus* cultures on casein agar

Culture No	Colony diameter	Proteolysis zone diameter	Proteolytic activity index
NCG1	9 mm	14	1.55
NCG2	10 mm	20	2
NCG3	9 mm	12	1.33
NCG4	9 mm	25	2.77
NCG5	10 mm	23	2.3
NCG6	11 mm	20	1.82
NCG7	10 mm	15	1.5
NCG8	9 mm	20	2.2
NCG9	9 mm	15	1.66
NCG10	10 mm	23	2.3
NCG11	10 mm	18	1.8
NCG12	10 mm	23	2.3
NCG13	10 mm	17	1.7
NCG14	10 mm	19	1.9
NCG15	10 mm	18	1.8
NCG16	8 mm	20	2.5
NCG17	9 mm	15	1.66
NCG18	10 mm	18	1.8
NCG19	10 mm	19	1.9
NCG20	10 mm	17	1.7
NCG21	9 mm	15	1.66
NCG22	9 mm	18	2
NCG23	10 mm	17	1.7
NCG24	10 mm	19	1.9
NCG25	9 mm	15	1.66

4.2.9.3.2. Proteolytic activity of coagulase negative *Staphylococcus* cultures on Gelatin agar

Culture No	Colony diameter	Proteolysis zone diameter	Proteolytic acivity Index
NCG1	9 mm	-ve	--
NCG2	9 mm	-ve	
NCG3	10 mm	-ve	
NCG4	9 mm	-ve	--
NCG5	9 mm	-ve	--
NCG6	10 mm	15mm	1.5
NCG7	8 mm	-ve	--
NCG8	9 mm	-ve	--
NCG9	10 mm	-ve	--
NCG10	10 mm	-ve	--
NCG11	8 mm	18mm	2.25
NCG12	9 mm	-ve	--
NCG13	9 mm	-ve	--
NCG14	10 mm	-ve	--
NCG15	9 mm	-ve	--
NCG16	9 mm	17mm	1.8
NCG17	8 mm	-ve	--
NCG18	10 mm	-ve	--
NCG19	10 mm	-ve	--
NCG20	9 mm	-ve	--
NCG21	9 mm	18mm	2
NCG22	10 mm	-ve	--
NCG23	9 mm	-ve	--
NCG24	9 mm	-ve	--
NCG25	9 mm	18mm	2

behavoiur was noted in the case of coagulase positive cultures as well (see Table 4.2.4.3.1 and 4.2.4.3.2. vide infra).

Bhat *et al,* (1990) has investigated the proteolytic activity of both coagulase positive and coagulase negative *Staphylococcus* cultures isolated from clinical cases using gelatin agar and they have found that 50% of the coagulase negative cultures were proteolytic. In the present study 100% of the cultures have exhibited proteolytic activity against casein but only 20% against gelatin and to that extent the findings vary.

4.2.9.4. Thermonuclease and phosphatase activity of coagulase negative *Staphylococcus* species isolated from fish and fishery products

Thermonuclease and phosphatase activities of the 25 coagulase negative *Staphylococcus* cultures from fish and fishery products were determined and the results are presented in Table 4.2.9.4. The data on coagulase reaction is also included in the Table for comparison. It can be seen that all the cultures were negative for their thermonuclease activity. However 17 out of the 25 cultures exhibited phosphatase activity. All the strains of *S.epidermidis* and *S.hysicus sub species chromogens* showed phosphatase activity while all the seven *S.simulans* and one of the two *S.warneri* were negative for phosphatase activity. In total, 17 out of the 25 (68%) of the coagulase negative cultures tested were capable of producing phosphatase enzyme. Langlois *et al,* (1990) have investigated the phosphatase activity of coagulase negative *Staphylococcus* of bovine and human origin and reported that this activity varied between 0-91% depending up on species. According to Bergy's manual of Systematic bacteriology (vol.2, Sneath *et al,* 1986) eleven out of the 19 species of *Staphylococcus* exhibit alkaline phosphatase activity (90^{+}). Bhat *et al,* (1990) in their studies on the biochemical characteristics of *Staphylococcus* cultures from clinical samples have found that 30% of coagulase negative *Staphylococcus* produced phosphatase.

4.2.9.4. Thermonuclease and Phosphatase activity of coagulase negative *Staphylococcus* cultures from fish and fishery products

Culture No	Coagulase test	Thermonuclease activity	Phosphatase activity
NCG1	-ve	-ve	-ve
NCG2	-ve	-ve	+ve
NCG3	-ve	-ve	+ve
NCG4	-ve	-ve	-ve
NCG5	-ve	-ve	+ve
NCG6	-ve	-ve	-ve
NCG7	-ve	-ve	+ve
NCG8	-ve	-ve	+ve
NCG9	-ve	-ve	-ve
NCG10	-ve	-ve	-ve
NCG11	-ve	-ve	-ve
NCG12	-ve	-ve	+ve
NCG13	-ve	-ve	+ve
NCG14	-ve	-ve	-ve
NCG15	-ve	-ve	+ve
NCG16	-ve	-ve	-ve
NCG17	-ve	-ve	+ve
NCG18	-ve	-ve	+ve
NCG19	-ve	-ve	+ve
NCG20	-ve	-ve	+ve
NCG21	-ve	-ve	+ve
NCG22	-ve	-ve	+ve
NCG23	-ve	-ve	+ve
NCG24	-ve	-ve	+ve
NCG25	-ve	-ve	+ve

4.2.9.5. Haemolytic activity of coagulase negative *Staphylococcus* cultures isolated from fish and fishery products

Haemolytic activity of the 25 coagulase negative *Staphylococcus* cultures were determined on human blood agar as described in the materials and methods section and the data are presented in Table 4.2.9.5. Two out of the 25 cultures, were positive for α-haemolytic activity, ten out of 25 were β-haemolytic and the rest non-haemolytic (γ). According to Bergy's manual of Systematic Bacteriology (Vol.2, 1986) the haemolytic activity of coagulase negative *Staphylococcus* are mostly negative or weak. Studies of Jassim *et al,* (1989) has also reported that haemolytic activity of coagulase negative staphylococci varied with the microorganism as well as species of blood source. In the present study human blood was used and many of the cultures have shown β-haemolytic activity on the blood agar. It is interesting to compare the haemolytic property of coagulase negative *Staphylococcus* with those of *S.aureus* isolated from fish and fishery products.60% of both coagulase positive and negative Staphylococci were β- haemolytic. In the case of α-haemolysis two out of 25 cultures in each group were found to be positive.

4.2.10. Antibiotic sensitivity of coagulase negative *Staphylococcus* cultures from fish and fishery products

The results of the antibiotic sensitivity tests of coagulase negative staphylococci are given in Table 4.2.10. A total of 12 antibiotics were used for the study. Nalidixic acid was the drug towards which 96% of the isolates showed resistance. Resistance towards other antibiotics was as follows:- Oxolinic acid (92%), Sulphamethaxazole (36%), Penicillin (32%) and Tetracycline (4%). None of the isolates showed resistance to Chloramphenicol, Neomycin, Vancomycin, Oxytetracycline, Methicillin, Erythromycin and Novobiocin.

So far, no work has been found reported on the antibiotic susceptibility of coagulase negative staphylococci isolated from fish and fishery products. The antibiotic

2.9.5. Haemolytic activity of coagulase negative *Staphylococcus* from fish and fishery products

Culture No	Haemolytic activity (Blood agar)	
	α	β
NCG1	-ve	+ve
NCG2	-ve	-ve
NCG3	-ve	-ve
NCG4	-ve	+ve
NCG5	-ve	-ve
NCG6	-ve	-ve
NCG7	-ve	-ve
NCG8	-ve	-ve
NCG9	-ve	+ve
NCG10	-ve	+ve
NCG11	-ve	+ve
NCG12	-ve	-ve
NCG13	-ve	-ve
NCG14	-ve	+ve
NCG15	-ve	+ve
NCG16	-ve	-ve
NCG17	-ve	-ve
NCG18	-ve	-ve
NCG19	-ve	-ve
NCG20	-ve	-ve
NCG21	+ve	+ve
NCG22	+ve	-ve
NCG23	+ve	+ve
NCG24	-ve	-ve
NCG25	-ve	+ve

4.2.10. Antibiotic sensitivity pattern of coagulase negative *Staphylococcus* cultures from fish and fishery products

Antibiotic used	Sensitive	%	Intermediate	%	Resistant	%
Chloramphenicol	25	100%	-	-	-	-
Neomycin	25	100%	-	-	-	-
Tetracycline	13	52%	11	44%	1	4%
Oxytetracycline	18	72%	7	28%	-	-
Nalidixic acid	1	4%	-	-	24	96%
Sulphamethaxazole	16	64%	-	-	9	36%
Oxolinic acid	2	8%	-	-	23	92%
Novobiocin	25	100%	-	-	-	-
Vancomycin	25	100%	-	-	-	-
Methicillin	25	100%	-	-	-	-
Erythromycin	18	72%	7	28%	-	-
Penicillin	17	68%	-	-	8	32%

susceptibility test carried out by Pinna *et al* (1991) in isolates from corneal infections showed that about 67.27% of the isolates were resistant to penicillin, 57.14% to tetracycline and 37.72% to erythromycin.

The multi resistant staphylococci have probably arisen by a succession of mutations conferring resistance to different strains that were at first only pencillin resistant. Plasmids that bear genes conferring resistance to an antibiotic may be transferred from a resistant to a sensitive strain by phage transduction and such transfer may be contributed to the acquisition of resistance by some strains. The predominance of multi resistant strain may be maintained by the wide spread use of anyone of the antibiotics to which it is resistant. (Al-Masudi *et al*, 1991)

4.2.11. Carbohydrate and Aminoacid utilization by coagulase negative *Staphylococcus* cultures from fish and fishery products

4.2.11.1. Utilization of Pentoses

Production of acid from three pentoses namely arabinose, ribose and xylose were studied with the 25 coagulase negative *Staphylococcus* cultures. The results are presented in Table 4.2.11.1. All the 25 cultures did not utilize either arabinose or xylose, while 22 out of the 25 (88%) cultures could ferment ribose to produce acid. According to Bergy's manual (vol.2, Sneath *et al* 1986) almost all the coagulase negative *Staphylococcus* cultures are negative for utilization of arabinose and xylose while fermentation of ribose varied between 11-89%. Langlois *et al,* (1990) has reported that none of the 855 *Staphylococcus* isolates of bovine and human origin consisting of *S.aureus*, *S.capitis*, *S.epidermidis*, *S.haemoluticus*, *S.warneri* and *S.simulans* could ferment arabinose and xylose, while 4.2% of *S.epidermidis* of human origin and 21.7% of bovine origin fermented ribose. Also 73.4% of *S.simulans*, 47.6% of *S.warneri* of bovine origin femented ribose. In the present study, except one culture each of *S.epidermidis*, *S.simulans*, *S.warneri*, all other isolates of the 25-coagulase negative *Staphylococcus*

4.2.11.1. Utilisation of Pentoses by coagulase negative *Staphylococcus* cultures from fish and fishery products

Culture No	Arabinose	Ribose	Xylose
NCG1	-ve	+ve	-ve
NCG2	-ve	+ve	-ve
NCG3	-ve	+ve	-ve
NCG4	-ve	+ve	-ve
NCG5	-ve	+ve	-ve
NCG6	-ve	-ve	-ve
NCG7	-ve	+ve	-ve
NCG8	-ve	+ve	-ve
NCG9	-ve	+ve	-ve
NCG10	-ve	+ve	-ve
NCG11	-ve	+ve	-ve
NCG12	-ve	+ve	-ve
NCG13	-ve	+ve	-ve
NCG14	-ve	+ve	-ve
NCG15	-ve	+w	-ve
NCG16	-ve	+ve	-ve
NCG17	-ve	+ve	-ve
NCG18	-ve	+ve	-ve
NCG19	-ve	-ve	-ve
NCG20	-ve	+ve	-ve
NCG21	-ve	+ve	-ve
NCG22	-ve	+ve	-ve
NCG23	-ve	-ve	-ve
NCG24	-ve	+ve	-ve
NCG25	-ve	+ve	-ve

isolates utilized ribose. It is interesting to note that coagulase positive *S.aureus* cultures also showed similar pattern of utilization of three pentoses.

4.2.11.2. Utilization of hexoses by coagulase negative *Staphylococcus* cultures from fish and fishery products

In Table 4.2.11.2. the results of the utilization of 5 hexoses namely fructose, galactose, glucose, mannitol and mannose by 25 coagulase negative *Staphylococcus* cultures isolated from fish and fishery products are presented. All the 25 cultures produced acid without gas from fructose, galactose, glucose and mannose while only 18 out of 25 (72%) of the cultures could ferment mannitol.

According to Bergy's manual of Systematic Bacteriology, (Vol.2. Sneath *et al,* 1986) 11-89% of *S.epidermidis* and 100% of *S.simulans*, *S.hysicus.chromogens* and *S.warneri* are positive for fermentation of galactose. 100% of all the cultures were positive for the utilization of fructose and 100% of *S.epidermidis,* 11-89% of *S.simulans* and 100% of *S.hysicus chromogens* were fermentative for mannose. *S.epidermidis* were negative for the fermentation of mannitol, 11-89% of *S.warneri* were positive for fermentation of mannose while none of the *S.hysicus chromogens* could ferment mannitol.

Langlois *et al,* (1990) had studied utilization of hexoses like fructose, galactose, mannose and mannitol by different species of coagulase negative *Staphylococcus*. In their study fructose was utilized by all the cultures while 87.5% of *S.epidermidis* utilized galactose and mannitol. Mannose was utilized by 75% of *S.epidermidis* and 100% of *S.warneri*. The results obtained in the present study are almost in agreement with the Bergy's manual of Systematic bacteriology as well as the results of Langlois *et al,* (1990). It will be interesting to note that 100% of the coagulase positive *S.aureus* tested fermented all the five hexoses while except mannitol 100% of other hexoses were fermeted by all the coagulase negative *Staphylococcus* isolated from fish and fishery products. Six out of the seven *S.epidermidis* cultures failed to ferment mannitol.

4.2.11.2. Utilisation of hexoses by coagulase negative *Staphylococcus* cultures from fish and fishery products

Culture No	Fructose	Galactose	Glucose	Mannitol	Mannose
NCG1	+ve	+ve	+ve	+ve	+ve
NCG2	+ve	+ve	+ve	-ve	+ve
NCG3	+ve	+ve	+ve	+w	+ve
NCG4	+ve	+ve	+ve	+ve	+ve
NCG5	+ve	+ve	+ve	+ve	+ve
NCG6	+ve	+ve	+ve	+ve	+ve
NCG7	+ve	+ve	+ve	+w	+ve
NCG8	+ve	+ve	+ve	+w	+ve
NCG9	+ve	+ve	+ve	+ve	+ve
NCG10	+ve	+ve	+ve	+ve	+ve
NCG11	+ve	+ve	+ve	+ve	+ve
NCG12	+ve	+ve	+ve	+w	+ve
NCG13	+ve	+ve	+ve	+w	+ve
NCG14	+ve	+ve	+ve	+ve	+ve
NCG15	+ve	+ve	+ve	+ve	-ve
NCG16	+ve	+ve	+ve	+ve	+ve
NCG17	+ve	-ve	+ve	-ve	+ve
NCG18	+ve	+ve	+ve	+w	+ve
NCG19	+ve	-ve	+ve	+ve	-ve
NCG20	+ve	+ve	+ve	+ve	+ve
NCG21	+ve	+ve	+ve	-ve	+ve
NCG22	+ve	+ve	+ve	-ve	+ve
NCG23	+ve	+ve	+ve	-ve	+ve
NCG24	+ve	+ve	+ve	-ve	+ve
NCG25	+ve	+ve	+ve	-ve	+ve

4.2.11.3. Utilization of disaccharides by coagulase negative *Staphylococcus* isolated from fish and fishery products

Five disaccharides namely D-cellobiose, lactose, maltose, sucrose and trehalose were used for studying their utilization by the 25 coagulase negative *Staphylococcus* cultures from fish and fishery products and the results are presented in 4.2.11.3. All the 25 cultures fermented lactose, maltose and sucrose with the production of acid while 13 out of the 25 cultures fermented trehalose. None of the cultures could ferment cellobiose.

According to Bergy's manual of Systematic Bacteriology, none of the coagulase negative Staphylococci fermented D-cellobiose while sucrose is fermented by *S.capitis*, *S.warneri*, *S.simulans* and *S.hysicus chromogens*. In the case of maltose all the *S.epidermidis*, *S.warneri*, *S.simulans* fermented maltose, while only 11-89% of the *S.hysicus chromogens* fermented maltose. In the case of trehalose *S.epidermidis* are negative for fermentation. *S.warneri*, *S.simulans*, *S.hysicus chromogens* showed 90% fermentation.

Studies by Langlois *et al* (1990) has also reported similar results. The findings in the present study are almost in agreement with these reports.

4.2.11.4. Utilization of trisaccharides and sugar derivatives by coagulase negative *Staphylococcus* from fish and fishery products

Two trisaccharides namely raffinose, melezitose and a sugar derivatives salicin (hydroxymethyl phenyl glucose) were used for the study. The data are presented in Table 4.2.11.4. None of the coagulase negative *Staphylococcus* cultures fermented raffinose and salicin but two cultures namely *S.hysicus chromogens* and one untypable coagulase negative *Staphylococcus* fermented melizitose with the production of acid.

Studies on the utilization of trisaccharides by coagulase negative staphylococci are very scanty. Report from Langlois *et al*, (1990) regarding utilization of melezitose and salicin in the case of coagulase negative *Staphylococcus* of bovine and human origin is available. They found that 4.3% of *S.epidermis* and 9.5% of *S.warneri* of human origin

4.2.11.3. Utilisation of disaccharides by coagulase negative *Staphylococcus* cultures from fish and fishery products

Culture No	D-Cellobiose	Lactose	Maltose	Sucrose	D-Trehalose
NCG1	-ve	+ve	+w*	+ve	+ve
NCG2	-ve	+ve	+w	+ve	-ve
NCG3	-ve	+ve	+w	+ve	+ve
NCG4	-ve	+ve	+w	+ve	+ve
NCG5	-ve	+ve	+w	+ve	-ve
NCG6	-ve	+ve	+w	+ve	+ve
NCG7	-ve	+ve	+w	+ve	-ve
NCG8	-ve	+ve	+w	+ve	+ve
NCG9	-ve	+ve	+w	+ve	+ve
NCG10	-ve	+ve	+w	+ve	+ve
NCG11	-ve	+ve	+w	+ve	+ve
NCG12	-ve	+ve	+w	+ve	+ve
NCG13	-ve	+ve	+w	+ve	+ve
NCG14	-ve	+ve	+w	+ve	-ve
NCG15	-ve	+ve	+w	+ve	-ve
NCG16	-ve	+ve	+w	+ve	+ve
NCG17	-ve	-ve	+d**	+ve	-ve
NCG18	-ve	+ve	+w	+ve	+ve
NCG19	-ve	-ve	+w	+ve	+ve
NCG20	-ve	+ve	+w	+ve	-ve
NCG21	-ve	+ve	+w	+ve	-ve
NCG22	-ve	+ve	+w	+ve	-ve
NCG23	-ve	+ve	+w	+ve	-ve
NCG24	-ve	+ve	+w	+ve	-ve
NCG25	-ve	+ve	+w	+ve	-ve

+w = weak reaction. +d = delayed reaction

2.11.4. Utilisation of trisaccharides and sugar derivatives by coagulase negative *Staphylococcus* cultures from fish and fishery products

Culture No	Melezitose	Raffinose	Salicin
NCG1	-ve	-ve	-ve
NCG2	-ve	-ve	-ve
NCG3	-ve	-ve	-ve
NCG4	-ve	-ve	-ve
NCG5	-ve	-ve	-ve
NCG6	-ve	-ve	-ve
NCG7	-ve	-ve	-ve
NCG8	-ve	-ve	-ve
NCG9	-ve	-ve	-ve
NCG10	-ve	-ve	-ve
NCG11	-ve	-ve	-ve
NCG12	-ve	-ve	-ve
NCG13	-ve	-ve	-ve
NCG14	-ve	-ve	-ve
NCG15	-ve	-ve	-ve
NCG16	-ve	-ve	-ve
NCG17	+ve	-ve	-ve
NCG18	-ve	-ve	-ve
NCG19	+ve	-ve	-ve
NCG20	-ve	-ve	-ve
NCG21	-ve	-ve	-ve
NCG22	-ve	-ve	-ve
NCG23	-ve	-ve	-ve
NCG24	-ve	-ve	-ve
NCG25	-ve	-ve	-ve

and 0.8% of *S.chromogens* of bovine origin could utilize salicin. Melezitose was utilized by 29.2% of *S.epidermidis* of human origin and 8.7% of bovine origin. 4.8% of *S.warneri* showed weak utilization of melezitose. In the present study, none of *S.epidermidis* was found to utilize salicin or melezitose. It is interesting to note that none of the 25 coagulase positive *S.aureus* could ferment melezitose, salicin or raffinose.

4.2.11.5. Amino acid utilization by coagulase negative *Staphylococcus* from fish and fishery products

Utilization of arginine, histidine, lysine and ornithine by the 25 coagulase negative *Staphylococcus* cultures are presented in Table 4.2.11.5. It can be seen that all the cultures hydrolyzed arginine while none of the cultures could decarboxylate histidine, lysine or ornithine.

According to Bergy's manual of Systematic Bacteriology, majority of the strains of *S.epidermidis, S.warneri, S.simulans* and *S.hysicus chromogens* utilized arginine with the release of ammonia. No report has been found for reference for utilization of lysine, histidine and ornithine by coagulase negative *Staphylococcus*. It is interesting to note that *S.aureus* also showed similar utilization pattern of all these amino acids.

4.2.12. Enterotoxin production and factors affecting enterotoxigenesis by *S.aureus* isolated from fish and fishery products

S.aureus is not only pathogenic but also toxigenic. They are capable of producing enterotoxins in food. Those who consume the food containing the preformed toxins suffer from food poisoning. Generally four types of enterotoxins are produced by *S.aureus* strains viz: Staphylococcal entero toxin A (SEA), Staphylococcal entero toxin B (SEB), Staphylococcal entero toxin C (SEC) and Staphylococcal entero toxin D (SED). Many more Staphylococcal enterotoxins have been recognized based on immunological properties of enterotoxins produced by various strains. As of now enterotoxins up to SEO have been reported in literature. However from the food poisoning stand point, only

4.2.11.5. Utilization of Amino acid by coagulase negative *Staphylococcus* cultures from fish and fishery products

Culture No	Amino acid used			
	Arginine	Histidine	Lysine	Ornithine
NCG1	+ve	-ve	-ve	-ve
NCG2	+ve	-ve	-ve	-ve
NCG3	+ve	-ve	-ve	-ve
NCG4	+ve	-ve	-ve	-ve
NCG5	+ve	-ve	-ve	-ve
NCG6	+ve	-ve	-ve	-ve
NCG7	+ve	-ve	-ve	-ve
NCG8	+ve	-ve	-ve	-ve
NCG9	+ve	-ve	-ve	-ve
NCG10	+ve	-ve	-ve	-ve
NCG11	+ve	-ve	-ve	-ve
NCG12	+ve	-ve	-ve	-ve
NCG13	+ve	-ve	-ve	-ve
NCG14	+ve	-ve	-ve	-ve
NCG15	+ve	-ve	-ve	-ve
NCG16	+ve	-ve	-ve	-ve
NCG17	+ve	-ve	-ve	-ve
NCG18	+ve	-ve	-ve	-ve
NCG19	+ve	-ve	-ve	-ve
NCG20	+ve	-ve	-ve	-ve
NCG21	+ve	-ve	-ve	-ve
NCG22	+ve	-ve	-ve	-ve
NCG23	+ve	-ve	-ve	-ve
NCG24	+ve	-ve	-ve	-ve
NCG25	+ve	-ve	-ve	-ve

enterotoxin A, B, C and D are important. Accordingly, in this study only SEA, SEB, SEC and SED are included.

4.2.12.1. Enterotoxin production by coagulase positive *S.aureus* strains

Enterotoxin production of the 25 strains of *S.aureus* strains isolated from fish and fishery products were studied by RPLA technique as described in the Materials and Methods section. Only enterotoxins A, B, C and D were screened and the results are presented in Table 4.2.12.1.

Only 19 out of the 25 *S.aureus* cultures (76%) produced enterotoxin. Five of the cultures produced SEA, 10 strains produced SEB and 9 cultures produced SEC. None of the cultures produced SED. Of these 19 cultures, 14 strains produced only a single enterotoxins, while five of them produced two different enterotoxins each.

The literature shows that the enterotoxin production potential among the *S.aureus* strain varies with the strain concerned (Genigeorgis, 1989; Mossel and van Netten, 1990; Bennet *et al,* 1986; Castro *et al,* 1986; Kenny *et al,* 1993).

Ruzickova, (1994) showed that enterotoxigenicity of isolates from dairy and bovine mammary glands ranged between 0-56%. The enterotoxin producing potential of *S.aureus* strains were also found to vary from 16-86% depending on source of strains (De Buyser *et al,* 1985; Isigidi *et al,* 1992 and Martin *et al,* 1992). Sanjeev *et al,* (1985) found that 88.46% of *S.aureus* strains from dried fish products were enterotoxigenic. Also they reported that (Sanjeev *et al*, 1986) 75.49% of *S.aureus* cultures isolated from frozen crab meat, cuttle fish, and prawn were enterotoxigenic. According to Mossel and van Netten, (1990) the variations in reported findings on the toxigenic potential of *S.aureus* strains could be due to the large variety of foods from which the strains are isolated and their variable ecological origin. A few authors (Isigidi *et al*,1992 and Mathieu *et al*, 1991) have shown that the enterotoxigenicity among *S.aureus* isolates varied according to their biovar. The most frequently enterotoxigenic strains were the strains of human origin.

4.2.12.1. Production of enterotoxins by *S.aureus* from fish and fishery products

Isolate No	Enterotoxin types			
	SEA	SEB	SEC	SED
CG1	- ve	- ve	- ve	- ve
CG2	- ve	- ve	- ve	- ve
CG3	- ve	- ve	- ve	- ve
CG4	- ve	- ve	- ve	- ve
CG5	- ve	- ve	- ve	- ve
CG6	- ve	+ve	- ve	- ve
CG7	+ve	- ve	+ve	- ve
CG8	- ve	- ve	- ve	- ve
CG9	- ve	+ve	- ve	- ve
CG10	- ve	+ve	- ve	- ve
CG11	+ve	+ve	- ve	- ve
CG12	- ve	- ve	+ve	- ve
CG13	+ve	- ve	- ve	- ve
CG14	+ve	+ve	- ve	- ve
CG15	+ve	+ve	- ve	- ve
CG16	- ve	- ve	+ve	- ve
CG17	- ve	+ve	- ve	- ve
CG18	- ve	+ve	- ve	- ve
CG19	- ve	+ve	- ve	- ve
CG20	- ve	- ve	+ve	- ve
CG21	- ve	- ve	+ve	- ve
CG22	- ve	- ve	+ve	- ve
CG23	- ve	- ve	+ve	- ve
CG24	- ve	+ve	+ve	- ve
CG25	- ve	- ve	+ve	- ve

4.2.12.2. Production of enterotoxin A (SEA) by *S.aureus* strains

The enterotoxin A production and the titre of the toxins produced by *S.aureus* strains are presented in Table.4.2.12.2. Out of the 25 *S.aureus* strains tested, only 5 strains (20%) produced staphylococcal enterotoxin A. The quantity of toxin produced by each culture in BHI broth at 37°C was assayed by the dilutions up to 1:128 dilutions serially, on the micro titre RPLA plates (Oxoid) as described under the Materials and Methods section. The *S.aureus* strains CG-7, CG-11, CG-13, CG-14 and CG15 produced SEA and the toxin produced was detectable by RPLA method up to 1:64 dilutions (titre). The minimum detection level of toxin by RPLA method (Oxoid) was 0.5ng/ microlitre ie, 0.5microgram/ml of the culture. Accordingly the quantity of SEA production by each of these cultures could be between 32microgram/ml-64microgram/ml (at least the toxin level could be 32microgram/ml).

The symptoms of food poisoning by *S.aureus* enterotoxins arises only if one injest a minimum of 100ng/g of food (0.1microgram/g of food) Varnam and Evans (1991). Since all the above five *S.aureus* strains could produce at least 32 microgram/ml of toxin at 37°C in 24 hours, they could cause food poisoning if permitted to grow and produce toxin in food. Sanjeev *et al,* (1986) reported that 30% of the *S.aureus* strains isolated from dried fish products were enterotoxin A producing. Sanjeev *et al,* (1985) and Sanjeev and Surendran, (1994) have found the predominance of enterotoxin A (SEA) producing *S.aureus* strains in frozen fish products as well. But in the present study only 5 out of 25 (20%) of the strains were found to produce SEA.

4.2.12.3. Production of Staphylococcal enterotoxin B (SEB) by *S.aureus* strains

The enterotoxin B (SEB) and the quantity of toxin produced by *S.aureus* strains isolated from fish and fishery products are presented in Table 4.2.12.3. Of the 25 *S.aureus* strains tested, 10 strains produced enterotoxin B in BHI culture at 37°C in 24 hours. The quantity of toxin produced was detectable up to 1:64 dilution of the culture broth.

4.2.12.2. Production of enterotoxin A by *S.aureus* cultures from fish and fishery products

Titre (dilutions)	Isolates producing SEA				
	CG 7	CG11	CG 13	CG 14	CG 15
1:1	+	+	+	+	+
1:2	+	+	+	+	+
1:4	+	+	+	+	+
1:8	+	+	+	+	+
1:16	+	+	+	+	+
1:32	+	+	+	+	+
1:64	+	+	+	+	+
1:128	-	-	-	-	-

4.2.12.3. Production of enterotoxin B by *S.aureus* strains from fish and fishery products

Titre (dilutions)	Isolates producing SEB									
	CG6	CG9	CG10	CG11	CG14	CG15	CG17	CG18	CG19	CG24
1:1	+	+	+	+	+	+	+	+	+	+
1:2	+	+	+	+	+	+	+	+	+	+
1:4	+	+	+	+	+	+	+	+	+	+
1:8	+	+	+	+	+	+	+	+	+	+
1:16	+	+	+	+	+	+	+	+	+	+
1:32	+	+	+	+	+	+	+	+	+	+
1:64	+	+	+	+	+	+	+	+	+	+
1:128	-	-	-	-	-	-	-	-	-	-

Considering the fact that the minimum detectable level of toxin by RPLA method (Oxoid) was 0.5 microgram/ml, each culture produced at least 32 microgram/ml of toxin in BHI broth in 24 hours. This quantity is well above the minimum food poisoning level of 100ng/g of food consumed.

Most of the reports on the toxin production by *S.aureus* cultures are from other types of foods and drinks. The reports from fishery products are scanty. Ng and Tay, (1993) has maid an extensive survey of the enterotoxin produced by *S.aureus* strains isolated from sweetened cold drinks, rice dishes, noodles, pastry and cutlets, hot dogs, sausages, meat items, ice creams, fruits and dessert. Only one isolate from cuttle fish was included in their study. They found that *S.aureus* culture isolated from these foods produced mostly A and B enterotoxins and occasionally SEC and SED. The *S.aureus* strains isolated from cuttle fish produced SEB. Holeckova *et al*, (2002) has reported that bacterial isolates from processed sheep, cheese, pista and sausages from Slovakia republic included enterotoxigenic *S.aureus* strains and 23.5% of those isolates produced Staphylococcal enterotoxin B (SEB).

Sanjeev *et al,* 1985 and 1986 investigated the enterotoxigenicity of of *S.aureus* strains from dried and frozen fish products. They found a very low incidence of Staphylococcal enterotoxins B (SEB) production among these isolates. Sanjeev and Surendran, (1994) found that only a very low percentage of *S.aureus* culture from frozen fish products were enterotoxin B producers. In the present study 40% of *S.aureus* strains from fish and fishery products have been found to produce SEB. Varadarajan and Nambudripad, (1982) have also reported the predominance of Staphylococcal enterotoxin B producers from milk products.

4.2.12.4. Production of enterotoxins C (SEC) by *S.aureus* strains isolated from fish and fishery products

Data on enterotoxins C by *S.aureus* strains isolated from fish and fish products are presented in Table 4.2.12.4. Out of the 25 *S.aureus* strains 9 produced (36%) SEC in BHI

4.2.12.4. Production of enterotoxin C (SEC) by *S.aureus* strains from fish and fishery products

Titre (dilutions)	Isolates producing SEC								
	CG7	CG12	CG16	CG20	CG21	CG22	CG23	CG24	CG25
1:1	+	+	+	+	+	+	+	+	+
1:2	+	+	+	+	+	+	+	+	+
1:4	+	+	+	+	+	+	+	+	+
1:8	+	+	+	+	+	+	+	+	+
1:16	-	+	+	+	+	+	+	+	+
1:32	-	+	+	+	+	+	+	+	+
1:64	-	+	+	+	+	+	+	+	+
1: 128	-	-	-	-	-	-	-	-	-

broth at 37°C in 24 hours. The toxin level was assayed up to 1:128 dilution and it has been found that up to 1:64 dilutions detectable level of the toxin was present in culture broth except the strain CG7 in which the toxin was detected upto 1: 16 dilution. Accordingly all the 8 strains produced at least 32microgram of toxin /ml of the culture broth. So as discussed earlier all these 10 cultures were capable of food poisoning.

As stated early, most of the reports on enteroxin production by *S.aureus* are for cultures isolated from vegetable, milk and meat products. Holeckova *et al,* (2002); Ng and Tay, (1993). Studies on toxigenicity of *S.aureus* strains from seafood are very limited. Sanjeev *et al,* (1985) found that 8.7% of *S.aureus* strain from dried fishery products produced SEC. They (Sanjeev *et al,* 1985) also found that 9.1% of *S.aureus* strains from frozen fish products could produce SEC. Sanjeev and Surendran, (1994) isolated Staphylococcal enterotoxins C producing cultures from 7.14% of the frozen fish samples analyzed. Sneha, (2004) found that majority of the *S.aureus* strains from fish and fish products were SEC producing. In the present study, 36% of the *S.aureus* strains studied have been found to produce SEC. Rajalakshmi and Rajyalakshmi, (1982) have found that the majority of *S.aureus* isolates recovered from bacterial food poisoning in India were found to produce SEC, either alone or in combination.

4.2.12.5. Enterotoxin D production by *S.aureus* strains from fish and fishery products

None of the coagulase positive *S.aureus* strains tested in this study could produce SED as shown in Table 4.2.12.1. However, other authors have found the occurance of SED producing *S.aureus* in different fish and fishery products. Sanjeev *et al,* (1985) found enterotoxins D as the 2nd dominating toxin, next to enterotoxin A in the *S.aureus* strains isolated from fishery products. A similar observation has been made by Sanjeev *et al,* (1986) in the case of *S.aureus* isolates from frozen fishery products. They found that 15.58% of the *S.aureus* isolates produced SED. Similarly Sanjeev and Surendran, (1994) have reported that 13.27% of the *S.aureus* isolates from frozen seafood were enterotoxins

D producers. However Sneha, (2004) could not isolate any SED producing *S.aureus* from fish and fishery products. The findings in the present study are also similar to her observations.

4.2.12.6. Multiple enterotoxins production by *S.aureus* strains isolated from fish and fishery products

Out of the 25 *S.aureus* strains tested, five cultures were found to produce multiple enterotoxins in BHI broth at 37°C in 24 hours. The data are presented in Table 4.2.12.6.

The *S.aureus* strain CG7 was found to produce SEA and SEC. The SEA was detected upto 1: 64 dilutions indicating that the quantities of these toxin produced were different while at least 32 microgram/ml of enterotoxin A was produced, the quantity of SEC produced was 8 microgram / ml in BHI broth.

S.aureus strains CG11, CG14 and CG15 were found to produce SEA and SEB together in BHI broth. Here also the quantity of toxin produced per ml of the BHI broth was 32 microgram/ml.

The *S.aureus* strain CG24 was found to produce SEB an SEC and both the toxins were detected up to a dilution of 1: 64. The quantity of each of these toxins produced were at least a minimum of 32 microgram/ml.

Enterotoxigenic *S.aureus* are known to produce more than one enterotoxin simultaneously in food (Ng and Tay, 1993; Wienke *et al,* 1993). Also Ng and Tay, (1993) have found *S.aureus* strains from various food products which could produce SEA,SEC and SEB together, SEA and SEC together and SEC and SED together. Sanjeev *et al,* (1985) reported 12 different combinations of enterotoxins production by *S.aureus* cultures isolated from frozen fish products from the cold storages of Cochin. Some of these *S.aureus* isolates produced 2 enterotoxins together, some three and some others four. Similar multiple enterotoxins production have been reported by Sanjeev *et al,* (1985) in the case of dried fishery products and Sanjeev and Surendran, (1994) in the case of *S.aureus* cultures from frozen fish products.

4.2.12.6. Multiple enterotoxins production by *S.aureus* strains from fish and fishery products

Culture no	Toxin types	Dilutions							
		1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
CG-7	**A**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**
	B	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	C	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**-ve**
	D	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CG-11	A	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**
	B	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**
	C	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	D	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CG-14	A	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**
	B	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**
	C	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	D	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CG 15	A	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**
	B	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**
	C	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	D	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CG 24	A	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	B	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**
	C	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**
	D	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

4.2.13. Enterotoxin production by coagulase negative *Staphylococcus* cultures isolated from fish and fishery products

Enterotoxigenicity of 25 coagulase negative *Staphylococcus* cultures isolated from fish and fishery products were studied in BHI broths as reported in the Materials and Methods section. The data are presented in table 4.2.13. Seven strains of *S.simulans*, seven strains of *S.hysicus chromogens*, seven strains of *S.epidermidis*, two strains of *S.warneri* and two untypable cultures were included in the list of cultures tested. The RPLA assay of enterotoxins A, B, C and D at 1:1 dilution of culture broth was made. At this dilution, toxin level of 0.5 microgram/ml could be detected. But it was found that none of the 25 cultures produced detectable levels of enterotoxins A, B, C or D. So it is concluded that coagulase negative *Staphylococcus* cultures isolated from fish and fish products in the present study were not capable of producing enterotoxins.

There are reports of the incidence of enterotoxigenic coagulase negative *Staphylococcus* from the hands of food handlers in Kuwait city (Udo *et al*, 1999). They found that 6% of the coagulase negative *Staphylococcus* produced enterotoxins A, B or C. Enterotoxin production by coagulase negative *Staphylococcus* from goat's milk and cheese was reported by Vernozy- Rozand *et al*, (1996). Valle *et al*, (1990) also have reported the isolation of enterotoxigenic coagulase negative strains of *Staphylococcus* from healthy goats. However, no such reports has been found in the case of coagulase negative *Staphylococcus* from seafood, either in India or elsewhere.

4.2.14. Studies on growth kinetics and enterotoxins production of *S.aureus* cultures isolated from fish and fishery products

The growth and toxin production of *S.aureus* are influenced by many factors viz; the size of the inoculam, the gaseous atmosphere, water activity of the growth medium, mineral ions and the media composition Smith *et al*, (1983). Based on the production characteristics the staphylococcal enterotoxins are differentiated into two groups (Youmans *et al*, 1980). Group 1 consists of SEB and SEC's, which are produced

4.2.13. Enterotoxigenicity of coagulase negative *Staphylococcus* cultures from fish and fishery products

Sl.No	Coagulase negative *Staphylococcus* spp	No of cultures Tested	Types of enterotoxin			
			A	B	C	D
1	*S.simulans*	7	-ve	-ve	-ve	-ve
2	*S.hysicus subsp.chromogens*	7	-ve	-ve	-ve	-ve
3	*S.eptdermidis*	7	-ve	-ve	-ve	-ve
4	*S.warneri*	2	-ve	-ve	-ve	-ve
5	*Untypable*	2	-ve	-ve	-ve	-ve

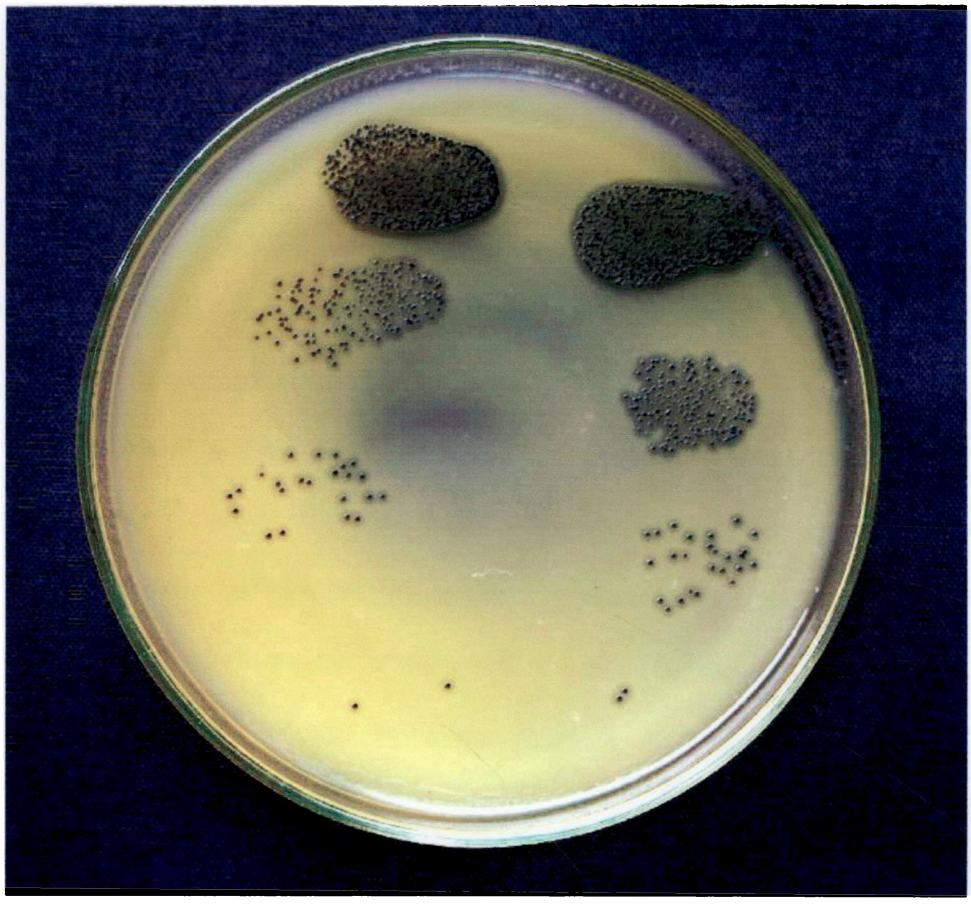


Fig.9. Enumeration of *Staphylococcus* by drop plate method

comparatively in larger quantities. Their production depends on cultural conditions as well as conditions of incubations. The 2nd group consists of SEA, SED and SEE, which are generally produced in smaller quantities. Their production is related to the growth of the strain, rather than the incubation conditions.

In order to study the growth kinetics and enterotoxin production, only representative strains were choosen from the toxigenic *S.aureus* strains from fish and fishery products, because of the enormous volume of work involved. A typical strain each from *S.aureus* cultures producing enterotoxins A, B and C were selected. Since there was no enterotoxin D producing *S.aureus* in the isolates the enterotoxin D producing strain could not be included in this investigation.

Effect of temperature, pH, water activity of the medium and salt on growth and toxin production in *in vitro* system, namely the BHI broth were studied. The details of experiments are presented in Materials and Methods section. The effect of the above physical and physico chemical factors on growth and toxin production of *S.aureus* strains were also studied in raw fish and shrimp tissue, as well as cooked fish and shrimp tissue.

4.2.14.1. Effect of temperture on growth and toxin production by enterotoxin A producing *S.aureus* strain (CG13)

The effect of six incubation temperatures, namely 0°C, 8°C, room temperature (28±2°C), 37°C, 45°C and 56°C on the growth and toxin production of the enterotoxin A producing *S.aureus* strain CG13 is presented in Table 4.2.14.1 and figure 4.2.14.1.

The inoculam size was almost kept at 120 cells/ ml media and the growth and toxin production were monitored at intervals of 7 hours, 24 hours, 48 hours and then at 8 days, 16 days, 24 days and 30 days as described in the materials and methods section. It is evident from the Table that at 0°C and 56°C the *S.aureus* culture did not grow. At 0°C there was a decline in the number of cells inoculated, a few cells survived in the medium even on 30th day while at 56°C the viability of the cells were not discernable even at the 7th hour of inoculation, indicating the death of the cells at that temperature at the initial

4.2.14.1. Effect of temperature on growth and toxin production by enterotoxin A (SEA) producing *S.aureus* strain CG13, from fish and fishery products

(Number of cells inoculated =120 cells/ml BHI broth)

Incubation Period	0°C		8°C		RT (28±2 °C)		37°C		45°C		56°C	
	No of cells/ ml	Toxin	No of cells/ml	Toxin	No of cells/ ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin
7 hr	100*	N.D	150*	N.D	$3.5x10^5$	N.D	$1.1x10^7$	Toxin	$2x10^7$	Toxin	No growth	N.D
24 hr	100*	,,	$3.34x10^3$	,,	$1.16x10^8$	Toxin	$4x10^8$	Toxin	$2.8x10^8$	Toxin	No growth	N.D
48 hr	100*	,,	$1.2x10^4$	,,	$1.8x10^9$	Toxin	$1.6x10^9$	Toxin	$8x10^8$	Toxin	No growth	N.D
192 hr (8 days)	50*	,,	$5x10^7$	,,	$2.5x10^9$	Toxin	$7.5x10^6$	Toxin	$1x10^6$	Toxin	No growth	N.D
384 hr (16 days)	50*	,,	$3.6x10^8$	,,	$2.1x10^8$	Toxin	$1.5x10^6$	Toxin	$5x10^4$	Toxin	No growth	N.D
576 (24 days)	50*	,,	$7.5x10^8$	,,	$2x10^7$	Toxin	$3x10^4$	Toxin	-	Toxin	No growth	N.D
720 (30 days)	50*	,,	$7x10^8$	,,	$1.2x10^7$	Toxin	$2x10^4$	Toxin	-	Toxin	N.D	N.D

* counts rounded of to nearest ten. N.D- Not Detected

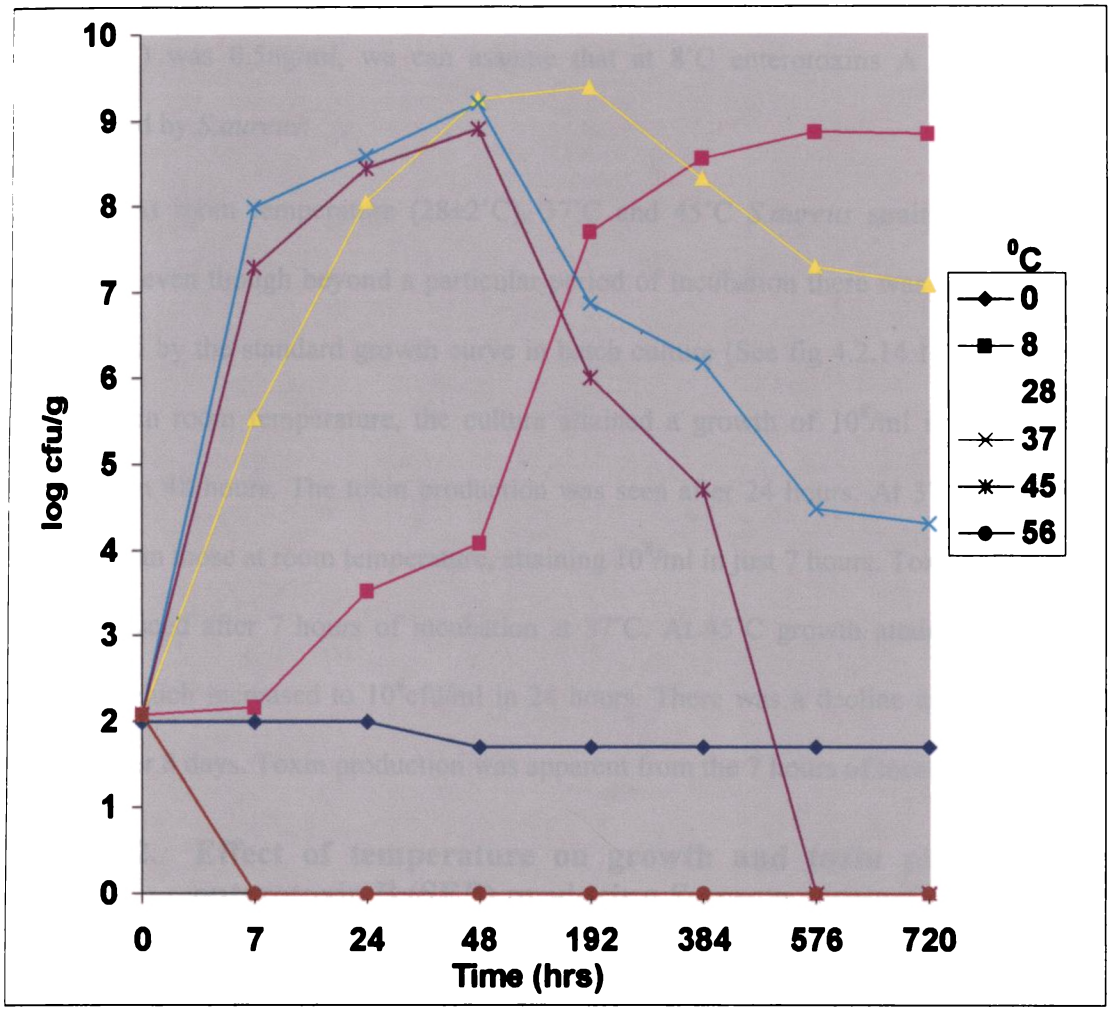


Fig 4.2.14.1. Effect of temperature on growth curve of SEA producing *S. aureus* strain CG13.

hours of incubation itself. At 8°C there was slow but study growth of *S.aureus* and the cell count reached 10^4 cfu/g in 48 hours and 10^8 cfu/g in 16 days; but no toxin was detected even on 30th day of incubation, even though the cell count has reached 10^8cfu/ml. Considering that the minimum detection level of toxin by RPLA method (Oxoid) was 0.5ng/ml, we can assume that at 8°C enterotoxins A (SEA) was not produced by *S.aureus*.

At room temperature (28±2°C), 37°C and 45°C *S.aureus* strains showed good growth, even though beyond a particular period of incubation there was a decline as we expected by the standard growth curve in batch culture (See fig 4.2.14.1). In the case of growth in room temperature, the culture attained a growth of 10^8/ml in 24 hours and 10^9/ml in 48 hours. The toxin production was seen after 24 hours. At 37°C growth was faster than those at room temperature, attaining 10^8/ml in just 7 hours. Toxin was found to be produced after 7 hours of incubation at 37°C. At 45°C growth attained 10^7/ml in 7 hours, which increased to 10^8cfu/ml in 24 hours. There was a decline in the number of cells after 8 days. Toxin production was apparent from the 7 hours of incubation itself.

4.2.14.2. Effect of temperature on growth and toxin production by enterotoxin B (SEB) producing *S.aureus* strain CG9

The effect of temperature on growth and toxin production by enterotoxins B producing *S.aureus* strain CG9 is presented in Table 4.2.14.2 and figure 4.2.14.2. At 0°C and 56°C the *S.aureus* strain did not show any growth. Though at 0°C the cells lost their viability slowly, even after one month of incubation few cells were still viable. But at 56°C no viable cell was detected even on the 7th hour of inoculation. At 8°C growth was gradual, attaining 10^7/ml on the 8th day and 10^9 cfu/ml on 16th day. However no toxin was detected even after 30 days of incubation.

At room temperature (28±2°C), 37°C and 45°C, there was good growth attaining 10^5/ml in 7 hours, in the case of room temperature, 10^6cfu/ml in the case of 37°C and

4.2.14.2. Effect of temperature on growth and toxin production by enterotoxin B (SEB) producing *S.aureus* strain CG9 from fish and fishery products

(No of cells inoculated = 160 cells/ml BHI broth)

Incubation Period	0°C		8°C		RT (28±2 °C)		37°C		45°C		56°C	
	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin
7 hr	150*	N.D	320	N.D	$2.72x10^5$	N.D	$5.2x10^6$	N.D	$5.6x10^6$	N.D	No growth	N.D
24 hr	50	N.D	$2.7x10^3$	N.D	$8x10^8$	Toxin	$1.2x10^9$	Toxin	$1.84x10^8$	Toxin	No growth	N.D
48 hr	50	N.D	$5x10^4$	N.D	$1.8x10^9$	Toxin	$1.15x10^9$	Toxin	$1.5x10^8$	Toxin	No growth	N.D
192 hr (8 days)	50	N.D	$6x10^7$	N.D	$9x10^8$	Toxin	10^9	Toxin	10^7	Toxin	No growth	N.D
384 hr (16 days)	50	N.D	$1.05x10^9$	N.D	$1.2x10^7$	Toxin	$2.55x10^8$	Toxin	$3x10^6$	Toxin	No growth	N.D
576 hr (24 days)	50	N.D	$2.15x10^9$	N.D	$5.5x10^5$	Toxin	$5.5x10^6$	Toxin	$6x10^4$	Toxin	No growth	N.D
720 hr (30 days)	50	N.D	$7x10^8$	N.D	$3x10^5$	Toxin	$5x10^5$	Toxin	100	Toxin	No growth	N.D

* counts rounded of to the nearest ten.

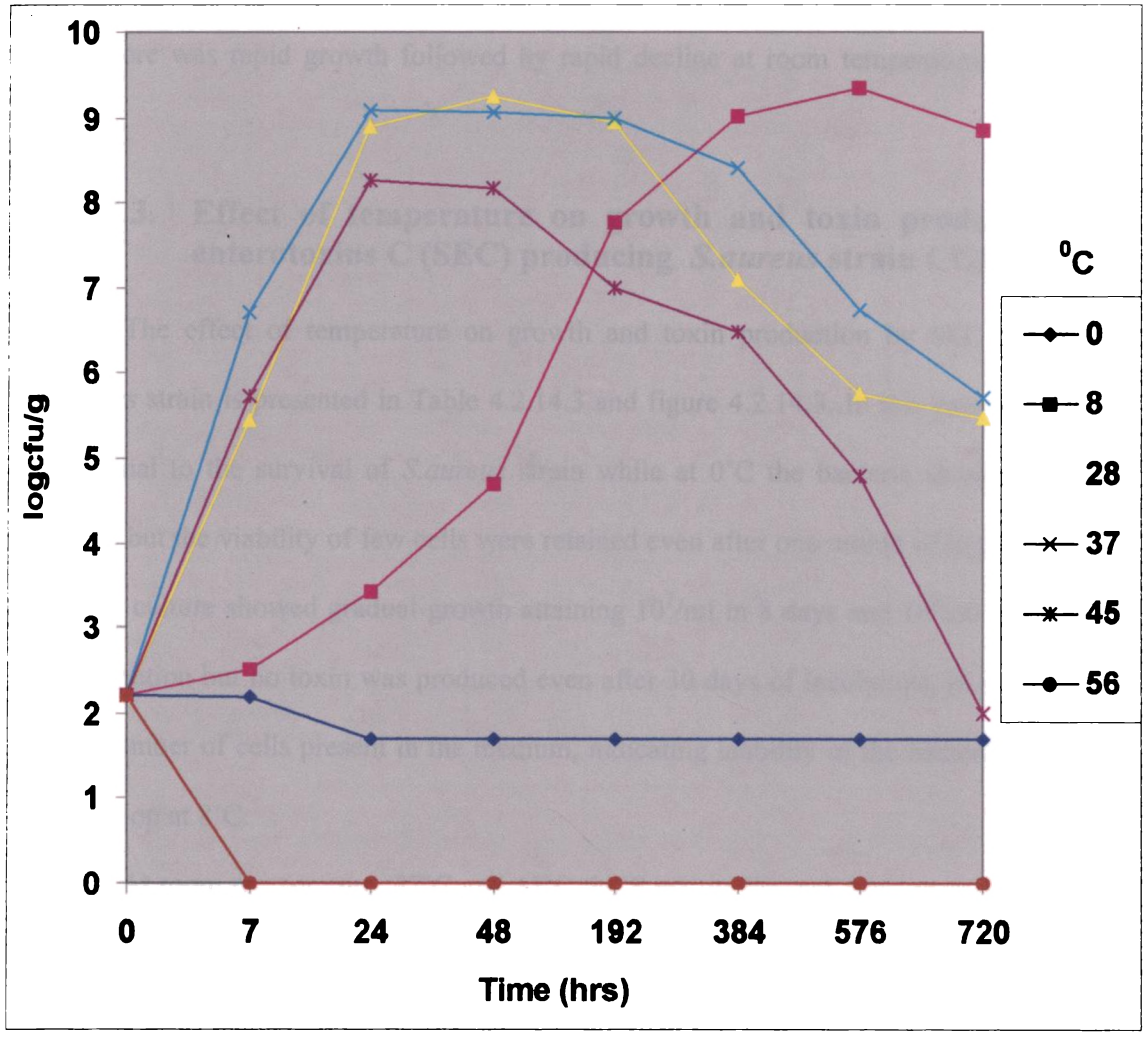


Fig.4.2.14.2. Effect of temperature on growth curve of SEB producing *S. aureus* strainCG9.

45°C, but no toxin was found to produce at that level of growth in all the three temperatures. In 24 hours of incubation, the population increased beyond 10^8/ml in all the three temperatures and the toxin was detected after 24 hours of incubation. The growth curve in fig 4.2.14.2 shows that while the bacteria continued to show steady growth at 8°C, there was rapid growth followed by rapid decline at room temperature, 37°C and 45°C.

4.2.14.3. Effect of temperature on growth and toxin production by enterotoxins C (SEC) producing *S.aureus* strain CG12

The effect of temperature on growth and toxin production by SEC producing *S.aureus* strain is presented in Table 4.2.14.3 and figure 4.2.14.3. In this case also, 56°C was lethal to the survival of *S.aureus* strain while at 0°C the bacteria showed gradual decline but the viability of few cells were retained even after one month of incubation. At 8°C the culture showed gradual growth attaining 10^7/ml in 8 days and 10^9/ml in 16 days of incubation but no toxin was produced even after 30 days of incubation, in spite of the large number of cells present in the medium, indicating inability of the bacteria for toxin production at 8°C.

At room temperature, 37°C and 45°C the *S.aureus* showed rapid growth in all the three temperatures. In 24 hours, the cells reached 10^8/ml or more and toxin was produced in all the three temperatures. In this case also no toxin was detected when the cell number was 10^6/ml at 37°C and 45°C. The growth curve in figure 4.2.14.3 shows similar behavior of this *S.aureus* culture, as in SEA and SEB producing strains as described above.

From Tables 4.2.14.1, 4.2.14.2 and 4.2.14.3 it is seen that all the three toxins producing strains of *S.aureus* isolated from fish and fish products produced enterotoxins in BHI broth after 24 hours of incubation at room temperature, 37°C and 45°C. In the case of SEA producing *S.aureus* CG13, toxin production was apparent at 7 hours of incubation at 37°C and 45°C when a cell population of 10^7/ml was exceeded. No toxin production was observed at an incubation temperature of 8°C even though the cell population was

4.2.14.3. Effect of temperature on growth and toxin production by enterotoxin C (SEC) producing *S.aureus* strain CG12 from fish and fishery products

(Number of cells inoculated = 100cells/ ml BHI broth)

Incubation Period	0°C		8°C		RT (28±2 °C)		37°C		45°C		56°C	
	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin
7 hr	80*	N.D	$3x10^2$	N.D	$1.72x10^5$	N.D	$2.8x10^6$	N.D	$5.8x10^6$	N.D	No growth	N.D
24 hr	50	N.D	$8x10^3$	N.D	$4.65x10^8$	Toxin	$1.15x10^9$	Toxin	10^8	Toxin	No growth	N.D
48 hr	50	N.D	$3x10^4$	N.D	$1.65x10^9$	Toxin	$2x10^9$	Toxin	$8x10^7$	Toxin	No growth	N.D
192 hr (8 days)	50	N.D	$4.32x10^7$	N.D	$3x10^9$	Toxin	$3.6x10^8$	Toxin	$5x10^7$	Toxin	No growth	N.D
384 hr (16 days)	50	N.D	$1.25x10^9$	N.D	$5.8x10^8$	Toxin	$5.5x10^5$	Toxin	$8x10^6$	Toxin	No growth	N.D
576 hr (24 days)	50	N.D	$9x10^9$	N.D	$9.5x10^7$	Toxin	$3.2x10^5$	Toxin	$7.5x10^4$	Toxin	No growth	N.D
720 hr (30 days)	50	N.D	$7.25x10^9$	N.D	$4x10^7$	Toxin	$1x10^5$	Toxin	$1.5x10^4$	Toxin	No growth	N.D

* counts rounded of to the nearest ten.

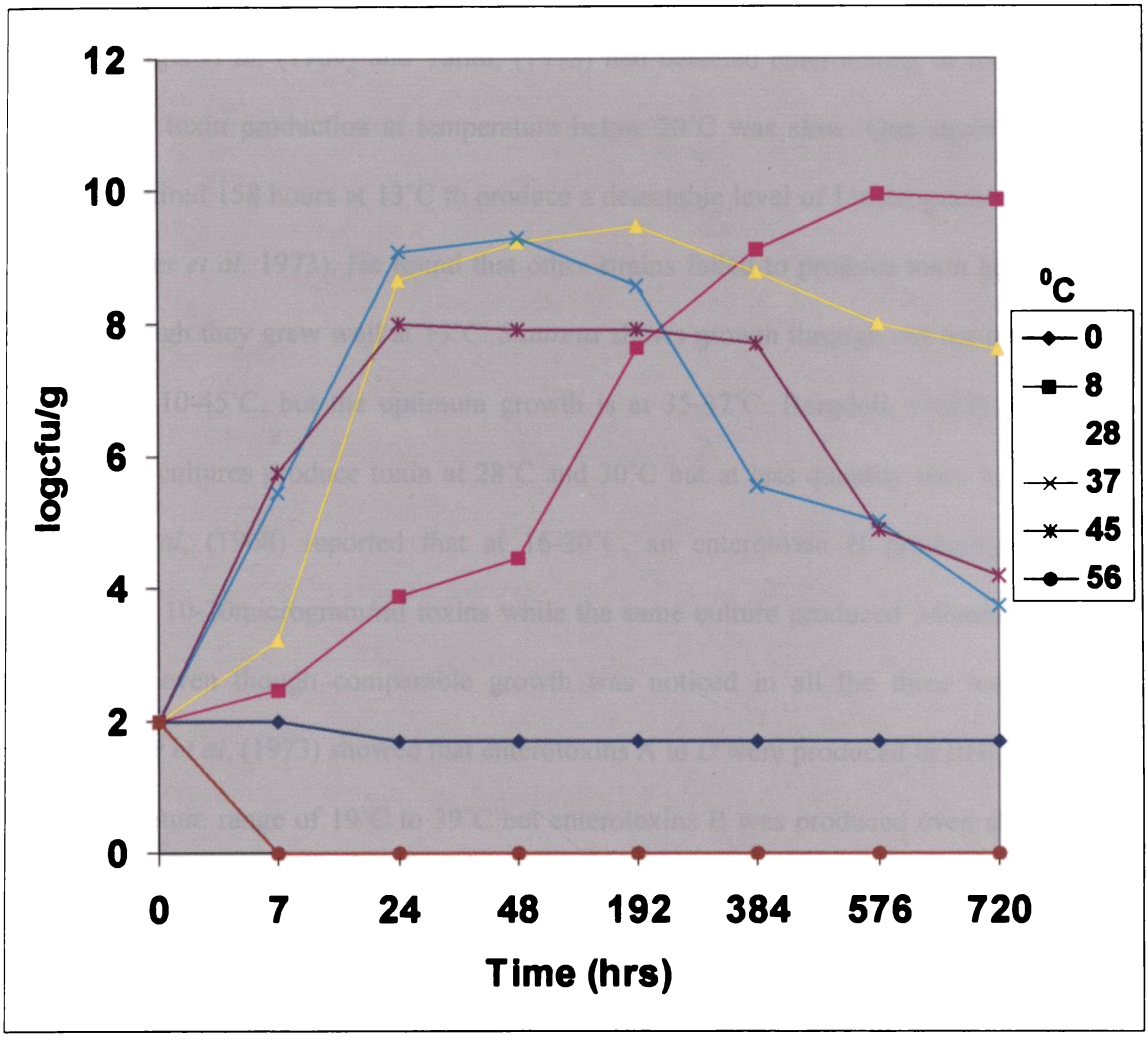


Fig. 4.2.14.3. Effect of temperature on the growth curve of SEC producing *S. aureus* strain CG12.

10^8 or above in the case of all the 3 entero toxigenic *S.aureus*, indicating that toxin production was not possible irrespective of high population at low temperature.

Angelotti *et al,* (1961) has reported that *S.aureus* can withstand low temperatures and grow at temperatures down to 7°C, but no toxin was produced at low temperatures. Genigeorgis *et al,* (1969) and Tatini, (1973) had detected enterotoxins in foods held at 10°C but toxin production at temperature below 20°C was slow. One strain producing SEB required 158 hours at 13°C to produce a detectable level of I microgram/ml of toxin (Scheusner *et al,* 1973). He found that other strains failed to produce toxin below 19°C, even though they grew well at 13°C. *S.aureus* shows growth through out the temperature range of 10-45°C, but the optimum growth is at 35-37°C. Bergdoll, (1989) found that *S.aureus* cultures produce toxin at 28°C and 30°C but at less quantity than at 37°C. Mc Lean *et al,* (1968) reported that at 16-20°C, an enterotoxin B producing *S.aureus* produced 10-20microgram/ml toxins while the same culture produced 340microgram/ml at 37°C, even though comparable growth was noticed in all the three temperatures. Scheusner *et al,* (1973) showed that enterotoxins A to D were produced in BHI broth over a temperature range of 19°C to 39°C but enterotoxins B was produced even at 45°C. He also found that enterotoxins B could be produced at temperature as low as 13°C. In the present study, enterotoxins A, B and C were produced at temperature ranging from 28±2°C to 45°C. The studies of Dietrich *et al,* (1972) showed that the maximum amount of enterotoxins B was produced at 37°C, 50% less at 35°C, 60% less at 40°C, 99% less at 20°C and none at 45°C. But in contrast to this result Vandenbosch *et al,* (1973) found maximum production of SEB and SEC at 40°C. In the present study, toxin production at all the 3 temperatures was almost the same by all the three types of toxigenic *S.aureus.*

A very large number of cells, $>2.8x10^8$ cells/ml must be present for the production of detectable level of toxin (Varnam and Evans, 1991). Lopez *et al,* (1993) has found that the minimum number of cells for the production of detectable level of enterotoxins at 37°C was 10^6/ml. In the present study, when the *S.aureus* population was 10^5/ml no toxin

was detected at room temperature, 37°C or 45°C but when the cell number was 10^7 toxins was detected at 45°C in the case of the enterotoxins A producing CG 13. In all other cases, toxin was detected at this toxigenic temperature only when the cell number was 10^8/ml. This observation is more or less is in agreement with the reports of Varnam and Evans (1991).

It can be concluded that enterotoxins production by *S.aureus* is not possible at low temperatures, particularly below 8°C. The toxin is generally produced at ambient temperatures (28±2°C) and above up to 45°C when the cell number was in the range of 10^7-10^8 cfu/ml.

4.2.14.4. Effect of incubation temperature on generation time of enterotoxigenic *S.aureus* strains

The generation time of the three enterotoxigenic *S.aureus* strains, namely CG13, CG9 and CG12 were calculated from the data presented in Table 4.2.14.1, 4.2.14.2 and 4.2.14.3. The results are presented in Table 4.2.14.4.

The generation time varied with the temperature of growth. There was no growth at 0°C and 56°C. At 8°C, generation time of SEA producing CG13 was 6.6 hours, SEB producing CG 9 was 7 hours and SEC producing CG12 was 4.43 hours. The corresponding values at room temperature were 36.5 minutes, 39.6 minutes and 46.2 minutes. At 37°C these values were respectively 0.31 hours (21 minutes), 0.60 hours (36 minutes) and 0.60 hours (36 minutes) while the corresponding values at 45°C were 0.4 hours (24 minutes), 36 minutes and 33 minutes. It can be noted that the generation time of enterotoxin A producing strain was lowest at 37°C. The generation time at 45°C was almost equal. In the case of the enterotoxins B producing strains lowest generatiom time of 36 minutes was obtained at 45°C while for SEC producing strains a generation time of 46.2 minutes was shown at RT (28±2°C). The manifestation of toxigenesis at 7 hours of incubation at 37°C and 45°C by enterotoxins A producing *S.aureus* strain is directly related to the very low generation time of 21-24 minutes at this temperatures.

4.2.14.4. Effect of Incubation temperature on Generation time of *S.aureus* from fish and fishery products

Culture No	Incubation temperature and generation time					
	0°C	8°C	RT	37°C	45°C	56°C
CG13 SEA)	No growth	6.6 hrs	36.5 minutes	21 minutes	24 minutes	No growth
CG9 (SEB)	„	7 hrs	39.6 minutes	36 minutes	36 minutes	„
CG12(SEC)	„	4.43 hrs	46.2 minutes	36 minutes	33 minutes	„

4.2.15. Effect of pH on growth and toxin production by enterotoxin producing *S.aureus* from fish and fishery products

Effect of pH on growth and enterotoxin production by Staphylococcal enterotoxin A producing *S.aureus* strain CG13, SEB producing *S.aureus* strain CG9 and SEC producing *S.aureus* strain CG12 were studied over a period of 30 days in BHI broth as described in Materials and Methods section. The results are presented in Table 4.2.15.1, 4.2.15.2 and 4.2.15.3 and figures 4.2.15.1, 4.2.15.2 and 4.2.15.3.

4.2.15.1. Effect of pH on growth and toxin production by SEA producing *S.aureus* strain CG13

The initial inoculam was 115 cells/ ml and the growth was monitored at intervals of 4 hours, 24 hours, 48 hours, 8 days, 16 days, 24 days and 30 days. From Table 4.2.15.1 it can be seen that at pH 3 and pH 12 the inoculam died out within 4 hours as evidenced by no growth in the sub culture medium. At pH 5, 7 and 9, *S.aureus* grew. In 24 hours the cell population in pH 5 reached 10^8/ml and the cell number was almost sustained at 10^8 cfu/ml ranges. After 16 days of incubation an expected decline in cell number was noticed. No toxin was detected at pH 5, inspite of the fact that the bacterial growth has exceeded 10^6 ml/mark indicating that the enterotoxin production was not possible at pH 5. At pH 7 in four hours, *S.aureus* population reached 10^5/ml and in 24 hours 10^7/ ml which showed further increase to 10^8/ml up to 8^{th} day and then a natural decline just like in any batch culture. From 24 hours onwards, enterotoxin A was detected in the culture broth. At pH 9 growth was slow compared with pH 7. In 24 hours the cell number reached 10^6/ml level but in that point, no toxin was detected in the culture broth. In fourty eight hours the *S.aureus* population reached 10^8/ml and also toxin was detected in the culture broth.

Figure 4.2.15.1 shows the growth curve of the *S.aureus* strain CG13 at different pH's. Only in pH 5, 7 and 9 positive growth curves were obtained. At pH 3 and 12 within 4 hours, the cells died out. The growth curves of the *S.aureus* strain at pH 5, 7 and 9 have the characteristic form of the growth curve of the typical bacterium in batch culture.

4.2.15.1.Effect of pH on growth and toxin production by enterotoxin A (SEA) producing *S.aureus* strain CG13 from fish and fishery products

(Number of cells inoculated = 115 cells/ ml BHI broth)

Incubation Period	pH 3		pH 5		pH 7		pH 9		pH 12	
	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin
4 hr	No growth	N.D	1050	N.D	$2x10^5$	N.D	800	N.D	No growth	N.D
24 hr	No growth	N.D	$6x10^8$	N.D	$9.5x10^7$	Toxin	$5x10^6$	N.D	No growth	N.D
48 hr	No growth	N.D	$3.5x10^7$	N.D	$9x10^8$	Toxin	$1.5x10^8$	Toxin	No growth	N.D
192 hr (8 days)	No growth	N.D	$2.05x10^8$	N.D	$8x10^8$	Toxin	$7.25X10^7$	Toxin	No growth	N.D
384 hr (16 days)	No growth	N.D	$9.75x10^7$	N.D	$6 x10^7$	Toxin	$2x10^7$	Toxin	No growth	N.D
576 hr (24 days)	No growth	N.D	$4.4 x10^5$	N.D	$2x10^6$	Toxin	$2.5x10^6$	Toxin	No growth	N.D
720 hr (30 days)	No growth	N.D	-	N.D	$3x10^5$	Toxin	$2x10^6$	Toxin	No growth	N.D

N.D- Not Detected

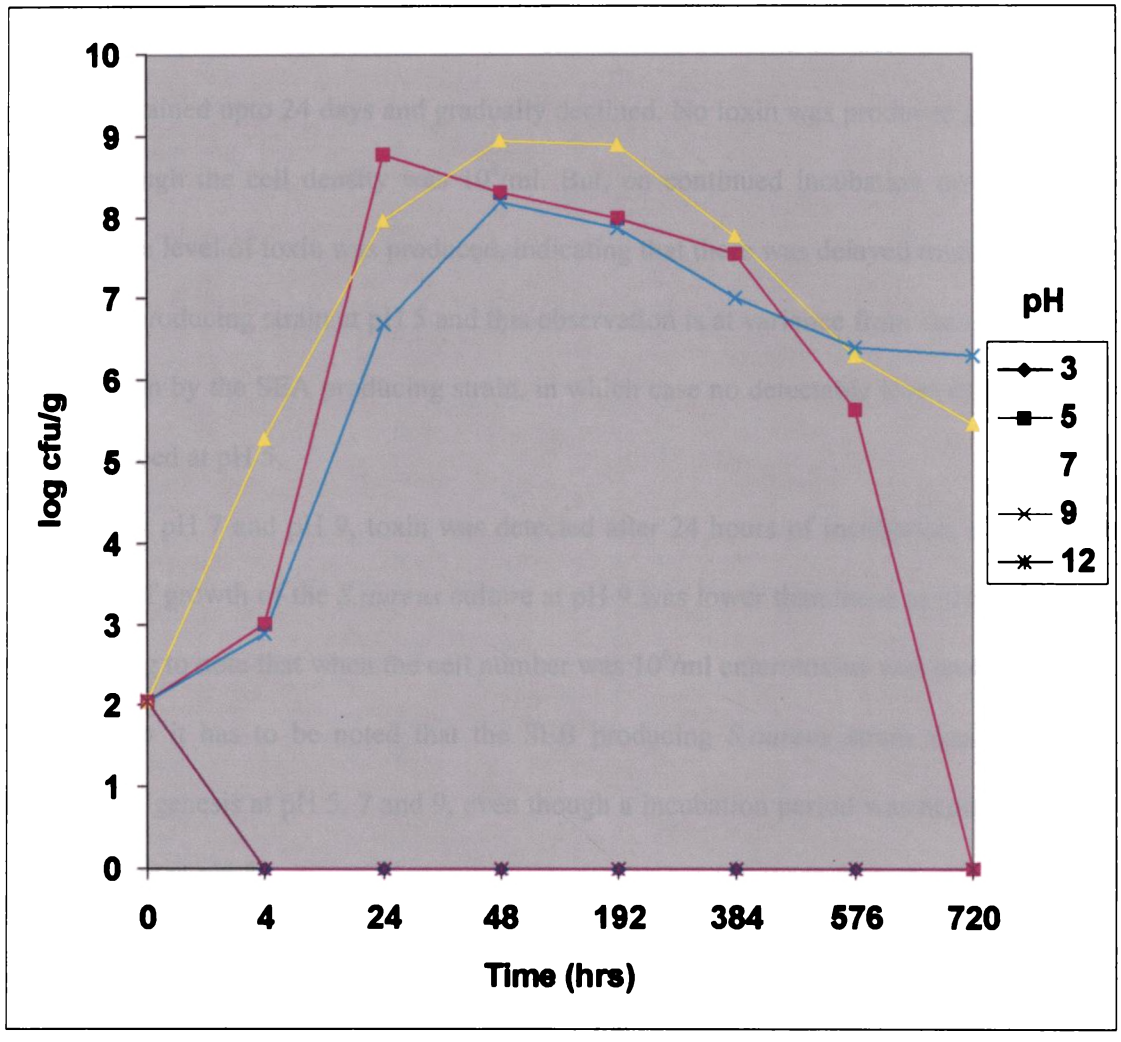


Fig.4.2.15.1. Effect of pH on the growth curve of SEA producing *S. aureus* strain CG13

4.2.15.2. Effect of pH on growth and toxin production by SEB producing *S.aureus* strain CG9

From Table 4.2.15.2 it can be seen that the *S.aureus* cells inoculated in BHI broth at pH 3 and 12 died out in 4 hours of incubation. At pH 5, 7 and 9 the culture showed growth. At pH 5 the population of the cells reaches 10^8 cells/ml after 24 hours, which were sustained upto 24 days and gradually declined. No toxin was produced up to 8 days, even though the cell density was 10^8/ml. But, on continued incubation up to 16 days, detectable level of toxin was produced, indicating that there was delayed toxin production by SEB producing strain at pH 5 and this observation is at variance from the case of toxin production by the SEA producing strain, in which case no detectable level of toxin could be produced at pH 5.

At pH 7 and pH 9, toxin was detected after 24 hours of incubation, even though the rate of growth of the *S.aureus* culture at pH 9 was lower than those at pH 7. It is also interesting to note that when the cell number was 10^6/ml enterotoxins was produced at pH 9. Further it has to be noted that the SEB producing *S.aureus* strain was capable of enterotoxigenesis at pH 5, 7 and 9, even though a incubation period was needed for toxin production at pH 5.

Figure 4.2.15.2 shows the growth levels of the SEB producing *S.aureus* strain CG9 at different pH's. At the acidic pH of 3 and highly alkaline pH of 12, there was no growth of inoculam in the BHI broth. The growth curves at pH 5, 7 and 9 had the standard form of a growth curve of bacterium in batch culture, as in the case of SEA producing *S.aureus* strain.

4.2.15.3. Effcet of pH on growth and enterotoxins production by SEC producing *S.aureus* strain CG12

The data presented in Table 4.2.15.3 shows that there was no growth of the cells in pH 3 and pH 12. Within 4 hours, the inoculam (95 cells/ml) died out. At pH 5 there was steady growth and the cell number reached 10^7 /ml in 24 hours and 10^8/ ml in 48

4.2.15.2. Effect of pH on growth and toxin production by enterotoxin B(SEB)producing *S.aureus* strain CG9Isolated from fish and fishery products

(Number of cells inoculated = 165 cells/ml)

Incubation Period	pH 3		pH 5		pH 7		pH 9		pH 12	
	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin
4 hr	No growth	N.D	950	N.D	$8x10^5$	N.D	800	N.D	No growth	N.D
24 hr	No growth	N.D	$2.05x10^8$	N.D	$9x10^8$	Toxin	$8x10^6$	Toxin	No growth	N.D
48 hr	No growth	N.D	$7x10^8$	N.D	$1x10^9$	Toxin	$7x10^7$	Toxin	No growth	N.D
192 hr (8 days)	No growth	N.D	$3.5x10^8$	N.D	$2.5x10^9$	Toxin	$6x10^7$	Toxin	No growth	N.D
384 hr (16 days)	No cells	N.D	10^8	Toxin	$8x10^8$	Toxin	$9x10^6$	Toxin	No growth	N.D
576 hr (24 days)	No growth	N.D	$9.5x10^7$	Toxin	$2x10^6$	Toxin	$3x10^6$	Toxin	No growth	N.D
720 hr (30 days)	No growth	N.D	$5x10^6$	Toxin	$2x10^4$	Toxin	$3x10^3$	Toxin	No growth	N.D

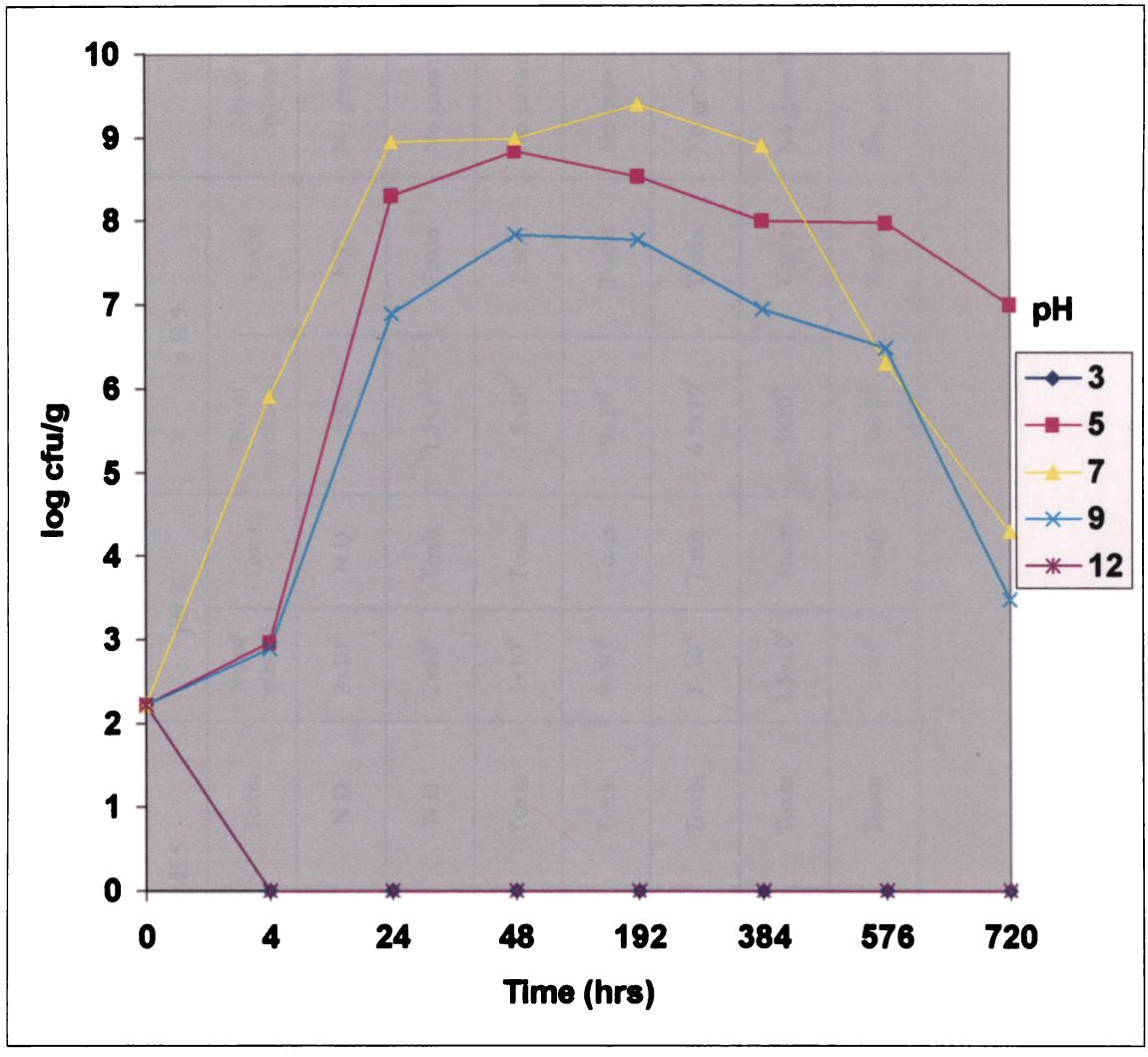


Fig.4.2.15.2. Effect of pH on the growth curve of SEB producing *S. aureus* strain CG9

4.2.15.3. Effect of pH on growth and toxin production by enterotoxin C (SEC) producing *S.aureus* strain CG12 Isolated from fish and fishery products

(Number of cells inoculated = 95cells/ml BHI broth)

Incubation Period	pH 3		pH 5		pH 7		p H 9		pH 12	
	No of cells	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin
4 hr	No growth	N.D	10^3	N.D	$3x10^5$	N.D	500	N.D	No growth	N.D
24 hr	No growth	N.D	$7.5x10^7$	N.D	$2x10^8$	Toxin	$1.5x10^6$	Toxin	No growth	N.D
48 hr	No growth	N.D	$4x10^8$	Toxin	$1x10^9$	Toxin	$1.5x10^8$	Toxin	No growth	N.D
192 hr (8 days)	No growth	N.D	$3.5x10^8$	Toxin	$9x10^8$	Toxin	$9x10^6$	Toxin	No growth	N.D
384 hr (16 days)	No growth	N.D	$9.5x10^7$	Toxin	$8x10^8$	Toxin	$4.5x10^6$	Toxin	No growth	N.D
576 hr (24 days)	No growth	N.D	$2x10^6$	Toxin	$2.5x10^6$	Toxin	$3x10^6$	Toxin	No growth	N.D
720hr (30 days)	No growth	N.D	$2x10^4$	Toxin	$2x10^4$	Toxin	$3x10^4$	Toxin	No growth	N.D

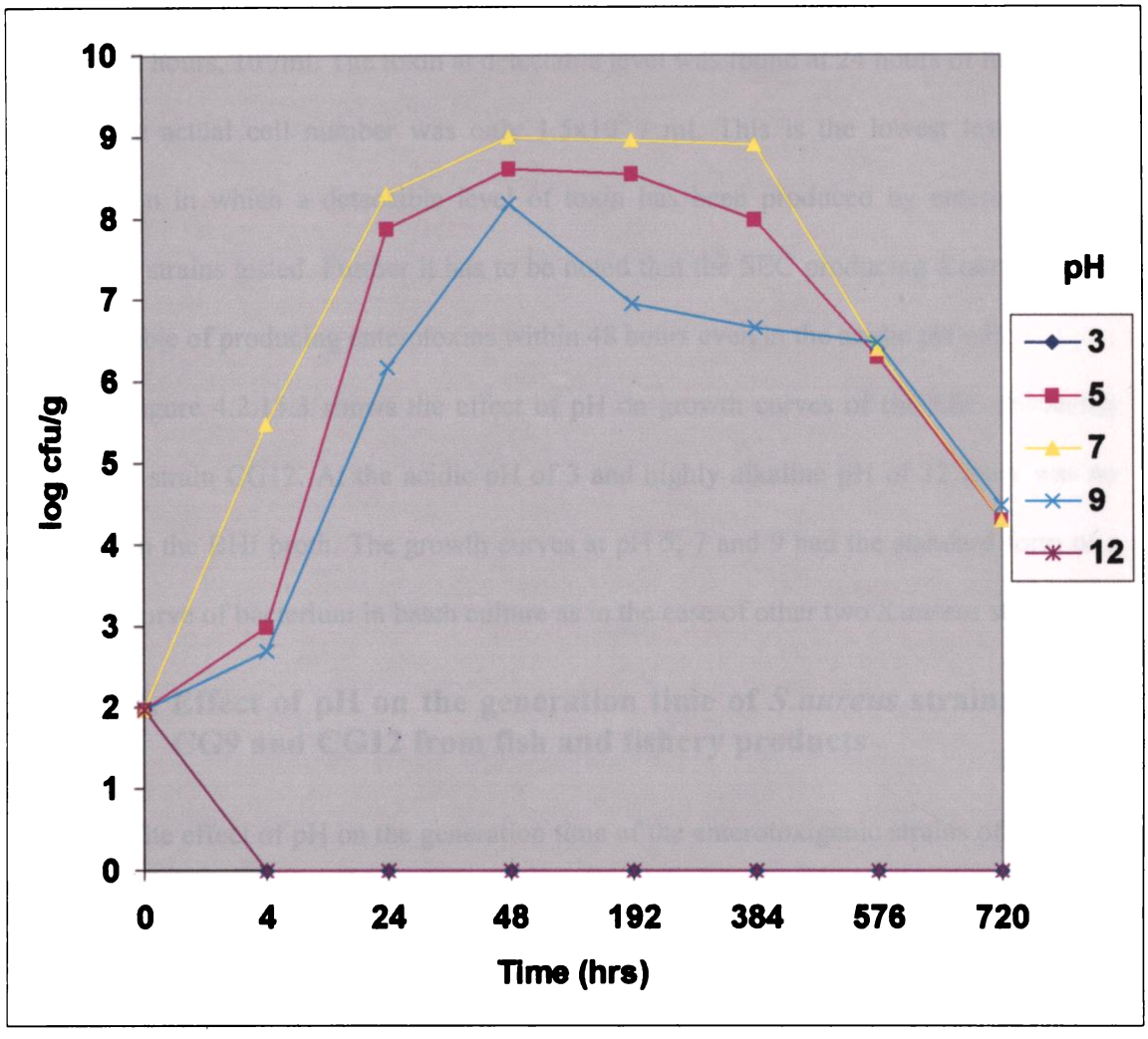


Fig. 4.2.15.3. Effect of pH on the growth curve of SEC producing *S. aureus* strain CG12

hours. No toxin was detected at 24 hours of growth, but detectable levels of toxin were produced after 48 hours onwards. At pH 7 and pH 9 there was steady growth and in 24 hours the cell number reached 10^8/ml with concurrent production of toxin. At pH 9 the initial level of growth was rather slow, but in 24 hours the cell number reached 10^6/ml and in 48 hours, 10^8/ml. The toxin at detectable level was found at 24 hours of incubation, when the actual cell number was only $1.5x10^6$ / ml. This is the lowest level of cell population in which a detectable level of toxin has been produced by enterotoxigenic *S.aureus* strains tested. Further it has to be noted that the SEC producing *S.aureus* strain was capable of producing enterotoxins within 48 hours even in the acidic pH of 5.

Figure 4.2.15.3 shows the effect of pH on growth curves of the SEC producing *S.aureus* strain CG12. At the acidic pH of 3 and highly alkaline pH of 12 there was no growth in the BHI broth. The growth curves at pH 5, 7 and 9 had the standard form of a growth curve of bacterium in batch culture as in the case of other two *S.aureus* strain.

4.2.15.4. Effect of pH on the generation time of *S.aureus* strains CG13, CG9 and CG12 from fish and fishery products

The effect of pH on the generation time of the enterotoxigenic strains of *S.aureus,* namely CG13, CG9 and CG12 were calculated from the data presented in Table 4.2.15.1, 4.2.15.2 and 4.2.15.3. The results are presented in Table 4.2.15.4. The generation time of *S.aureus* strains at pH 5 was found to be 1.25 hours for SEA producing strain CG13, 1.58 hours for SEB producing strain CG9 and 1.17 hours for SEC producing strain CG12. Similar generation times though on the higher side have been noticed for these cultures at pH 9; as well. At pH 7 the least generation times have been noticed namely 0.37 hours (22 minutes) for CG13, 0.34 hours (20 minutes) for CG9 and 19.6 minutes for CG12. This is on expected line because pH 7 is the optimum growth pH for *S.aureus* strains.

The pH of the culture media is very critical for the growth of bacteria. The pH at which a given strain will grow, depends on type of medium, the salt concentration, the

4.2.15.4. Effect of pH on Generation time of enterotoxigenic *S.aureus* cultures from fish and fishery products

Culture No	pH of growth media and generation time				
	pH 3	pH 5	pH7	pH 9	pH 12
CG13 (SEA)	No growth	1.25 hours	22 minutes	1.43 hours	No growth
CG9 (SEB)	No growth	1.58 hours	19.6 minutes	1.75 hours	No growth
CG12(SEC)	No growth	1.17 hours	20 minutes	1.67 hours	No growth

inoculam size and the aerobic atmosphere (Bergdoll, 1979). In order to study the pH on growth and toxin production by *S.aureus* strains, all factors other than pH were kept constant. A pH range of 3-12 have been selected, namely pH 3, 5, 7, 9 and 12. Most strains of *S.aureus* grow at pH value between 4.5 and 9.3 with optimum range of pH 7-7.5 but the pH, which supports the growth of *S.aureus*, may not favor enterotoxins production as well. Scheusner *et al,* (1973) found that the pH range for enterotoxin production by *S.aureus* was limited to pH 5.1 to pH 9.3. Reiser and Weiss, (1969) found that the low pH of 5.3 did not have a significant effect on enterotoxin A production of *S.aureus*. Even though a delay is caused at low pH, prolonged incubation up to 72 hours resulted in a higher toxin yield. Genigeorgis and Sadler, (1966) have made similar observations in the case of enterotoxin B production at pH 5. Scheusner *et al,* (1973) has reported that enterotoxin A, B, C and D were produced in a variety of food with pH values of 5.5-6.6, but not in foods with a pH below 5. Zehren and Zehren, (1968) has also found that in ham slices at a pH of 5.3 anaerobic production of SEB was possible. The finding in the present study is that enterotoxins B and C are produced in pH 5 even though delayed in the case of enterotoxin B. These findings are in general agreement with the published literature.

4.2.16. Effect of salt (NaCl) on growth and toxin production by *S.aureus* strains from fish and fish products

Salt (NaCl) is a critical requirment in culture media and usually 0.5% NaCl is an essential ingredient for the growth media of *S.aureus* cultures. The effect of salt on growth and enterotoxin production by *S.aureus* strains was determined in BHI broth. Salt at concentrations of 3%, 5%, 10%, 15%, 18% and 20% were incorporated in the BHI broth and a known number of viable *S.aureus* cells were inoculated and the growth and toxin production was determined as described in Materials and Methods section. The results are presented in Table 4.2.16.1, 4.2.16.2 and 4.2.16.3.

4.2.16.1. Effect of salt on growth and enterotoxin production by SEA producing *S.aureus* strain CG13

Table 4.2.16.1 gives the results of the study. The initial inoculam was 145 viable cells/ ml of culture medium. It may be noted that the *S.aureus* strains exhibited positive growth in all levels of NaCl in the medium viz; 3% - 20%, even though the rate of growth was lower at higher salt levels of 15% and above. At the salt levels of 3,5 and 10%, the culture attained a cell density of 10^8/ml in 24 hours. The cell number showed decline from 8 days onwards but enough number of live cells were present even after 30 days of incubation. Enterotoxin production was apparent from 24 hours of incubation at salt levels of 3, 5 and 10% . But only after 48 hours of incubation enterotoxin was detected at 15% salt level. At 18% and 20% salt levels, growth was very slow. After 16 days of incubation a cell count of $7.5x10^6$cfu/g was obtained with concurrent elaboration of toxin at 18% salt level. However no toxin was found to be produced at 20% of salt even after 30 days of incubation, in spite of the fact that the cell density of 10^7/ml was obtained after 16 days of incubation.

The effect of salt on the growth curve of SEA producing *S.aureus* strain CG13 is shown in figure 4.2.16.1. The growth curves of *S.aureus* strains at the salt level of 3, 5 and 10% are almost very similar in their form through out the period of incubation, while growth curves at salt level of 15, 18 and 20% have different gradients. Up to 48 hours they have an almost parallel shape to the base but after 48 hours sudden spurt in growth rate was observed.

4.2.16.2. Effect of salt (NaCl) on growth and enterotoxin production by SEB producing *S.aureus* strain CG9

The Table 4.2.16.2 presents the effect of salt in the growth media on growth and toxin production by SEB producing *S.aureus* strain CG9. The inoculam was 125 cells per ml of the growth medium. The salt level tested were 3%, 5%, 10%, 15%, 18% and 20% NaCl in BHI broth. At 3%, 5%, and 10% salt, *S.aureus* strain showed visible growth after

4.2.16.1. Effect of salt (NaCl) on growth and enterotoxin production by enterotoxin A (SEA) producing strain *S.aureus* strain CG 13 from fish and fishery products

(Number of cells inoculated = 145 cells/ ml BHI broth)

Incubation Period	3%		5%		10%		15%		18%		20%	
	No of cells	Toxin	No of Cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin
4 hr	$2.9x10^4$	N.D	$1x10^3$	N.D	900	N.D	200	N.D	200*	N.D	150*	N.D
24 hr	$2.6x10^8$	Toxin	$.55x10^8$	Toxin	$2.25x10^8$	Toxin	10^5	N.D	750	N.D	300*	N.D
48 hr	$3x10^8$	Toxin	$3.7x10^8$	Toxin	$1.8x10^8$	Toxin	$9.5x10^7$	Toxin	$1.95x10^3$	N.D	10^3	N.D
192 hrs (8 days)	$1.55x10^8$	Toxin	$7x10^7$	Toxin	$7.5x10^7$	Toxin	$8.5x10^7$	Toxin	$8x10^5$	N.D	$2x10^5$	N.D
384 hrs (16 days)	$2.5x10^7$	Toxin	$6x10^7$	Toxin	$6x10^7$	Toxin	$4 x10^6$	Toxin	$7.5x10^6$	Toxin	$1.5x10^7$	N.D
576 hrs (24 days)	$8.5x10^4$	Toxin	$2.75x10^5$	Toxin	$3.5x10^6$	Toxin	10^6	Toxin	$2.5x10^7$	Toxin	$1.5x10^6$	N.D
720 hrs (30 days)	$5x10^4$	Toxin	$2.25x10^5$	Toxin	$2.45x10^6$	Toxin	$1.25x10^5$	Toxin	$1.775x10^7$	Toxin	$1.5x10^5$	N.D

* Rounded of to the nearest ten

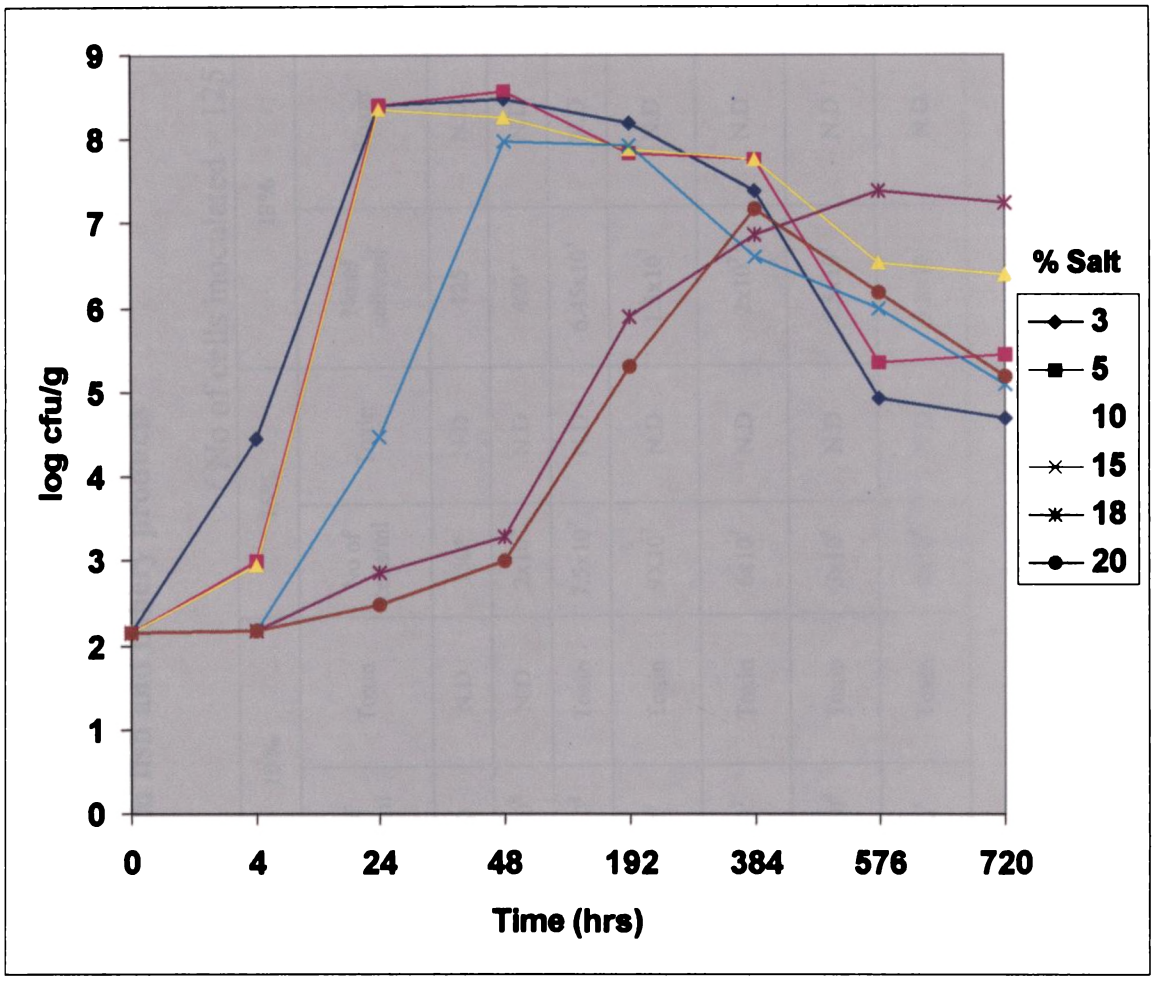


Fig. 4.2.16.1. Effect of salt on the growth curve of SEA producing *S. aureus* strain CG13

4.2.16.2. Effect of salt (NaCl) on growth and toxin production by enterotoxin B (SEB) producing *S.aureus* strain CG 9 from fish and fishery products

(No of cells inoculated = 125 cells/ ml BHI broth)

Incubation Period	3%		5%		10%		15%		18%		20%	
	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin	No of cells/ml	Toxin
4 hr	$5x10^4$	N.D	$2.5x10^3$	N.D	1500	N.D	150*	N.D	125	N.D	125	N.D
24 hr	$9.5x10^7$	Toxin	$7x10^8$	Toxin	$1.5x10^8$	N.D	$2x10^5$	N.D	400*	N.D	125	N.D
48 hr	$1.25x10^9$	Toxin	$2.5x10^8$	Toxin	$5.4x10^8$	Toxin	$7.5x10^7$	N.D	$6.45x10^3$	N.D	150	N.D
192 hr (8 days)	$2.5x10^7$	Toxin	$2.0x10^7$	Toxin	$2x10^7$	Toxin	$9X10^7$	N.D	$1.5x10^7$	N.D	$2x10^5$	N.D
384 hr (16 days)	$4.5x10^6$	Toxin	$1.5x10^7$	Toxin	$2 x10^7$	Toxin	$6x10^7$	N.D	$2x10^7$	N.D	$2.5x10^7$	N.D
576hr (24 days)	$1.5x10^5$	Toxin	$2.5 x10^6$	Toxin	$1.5x10^6$	Toxin	$3x10^6$	N.D	$7.5x10^7$	N.D	$1.25x10^8$	N.D
720 hr (30 days)	$3.25x10^4$	Toxin	$1.47x10^5$	Toxin	$3x10^5$	Toxin	$7x10^5$	N.D	$5.5x10^7$	N.D	$8.5x10^7$	N.D

* Rounded of to the nearest ten.

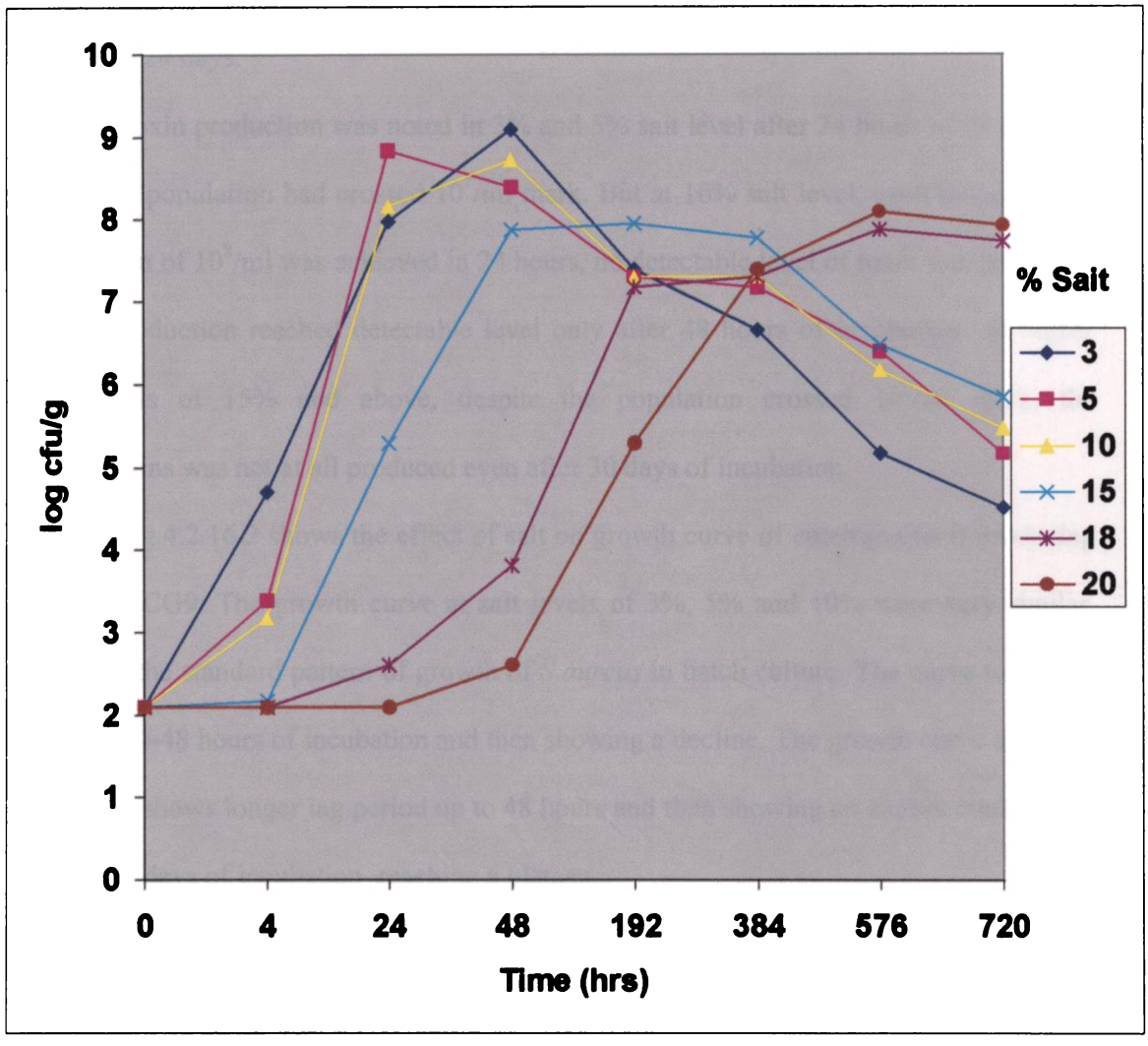


Fig. 4.2.16.2. Effect of salt on the growth curve of enterotoxin B producing *S. aureus* strain CG9

4 hours of incubation. But in higher salt levels, growth was found to be very slow. At 15% salt, growth attained 10^5/ml in 16 days and then slowly declined. At 18% and 20% salt, growth was very slow. In 48 hours growth reached 10^3/ml in 18% salt. In 20% salt, growth was apparent only after many days of incubation, reaching 10^5/ml in 8 days and 10^8/ml in 24 days.

Toxin production was noted in 3% and 5% salt level after 24 hours of incubation, when the population had crossed 10^7/ml mark. But at 10% salt level, even though a cell population of 10^8/ml was achieved in 24 hours, no detectable level of toxin was produced. Toxin production reached detectable level only after 48 hours of incubation. At higher salt levels of 15% and above, despite the population crossed 10^7/ml mark, the enterotoxins was not at all produced even after 30 days of incubation.

Fig.4.2.16.2 shows the effect of salt on growth curve of enterotoxins B producing *S.aureus* CG9. The growth curve at salt levels of 3%, 5% and 10% were very similar, showing the standard pattern of growth of *S.aureus* in batch culture. The curve reached a peak at 24-48 hours of incubation and then showing a decline. The growth curve at higher salt level shows longer lag period up to 48 hours and then showing an exponential growth for many days of incubation, reaching a plateau.

4.2.16.3. Effect of salt on growth and toxin production by SEC producing *S.aureus* CG12

The effect of salt on growth and toxin production of SEC producing *S.aureus* strain CG12 is presented in Table 4.2.16.3. The inoculam size was 110 cells/ml of growth medium. At 3%, 5%, and 10% salt levels growth was discernable after 4 hours and attained a growth level of above 10^8/ml in 24 hours, while growth was on a slower pace in 15% salt level reaching only 10^5/ml in 24 hours. At 18% and 20% salt levels growth was still slower reaching, 10^3/ml in 48 hours in the case of 18% salt and 800/ml in the case of 20% salt. However they exhibited a higher cell count above 10^7/ml after 8 days at 18% salt level and after 16 days in the case of 20% salt level.

4.2.16.3. Effect of salt (NaCl) on growth and toxin production by enterotoxin C (SEC) producing *S.aureus* strain CG 12 from fish and fishery products

(Number of cells inoculated = 110 cells/ ml BHI broth)

Incubation Period	3%		5%		10%		15%		18%		20%	
	No of cells	Toxin	No of cells Cfu/g	Toxin	No of cells	Toxin	No of cells	Toxin	No of cells	Toxin	No of cells	Toxin
4 hr	$7.7x10^3$	No Toxin	$2.2 x10^3$	N.D	1500	N.D	250	N.D	140*	N.D	130*	N.D
24 hr	$2.9x10^9$	Toxin	$3.9x10^8$	Toxin	$2x10^8$	N.D	$6.75x10^5$	N.D	250*	N.D	200*	N.D
48 hr	$3.75x10^9$	Toxin	$5.25x10^8$	Toxin	$3.375x10^8$	Toxin	$9.25x10^7$	Toxin	$3.2x10^3$	N.D	800	N.D
192 hr (8 days)	10^8	Toxin	$8.5x10^7$	Toxin	$9x10^7$	Toxin	$1x10^8$	Toxin	$1.2x10^7$	N.D	$2x10^5$	N.D
384 hr (16 days)	$9.5x10^7$	Toxin	$6.25 x10^7$	Toxin	$5.5x10^7$	Toxin	$3 x10^7$	Toxin	$4.5x10^7$	N.D	$4.5x10^7$	N.D
576hrs (24 days)	$8.25x10^5$	Toxin	$6x10^7$	Toxin	$1.25x10^7$	Toxin	$2.25x10^7$	Toxin	$1.5x10^7$	N.D	$3.5x10^7$	N.D
720hrs(30 days)	$3.5x10^4$	Toxin	$2.5x10^7$	Toxin	10^6	Toxin	$4.5x10^6$	Toxin	$1.25x10^7$	N.D	$3x10^7$	N.D

* Rounded of to the nearest ten.

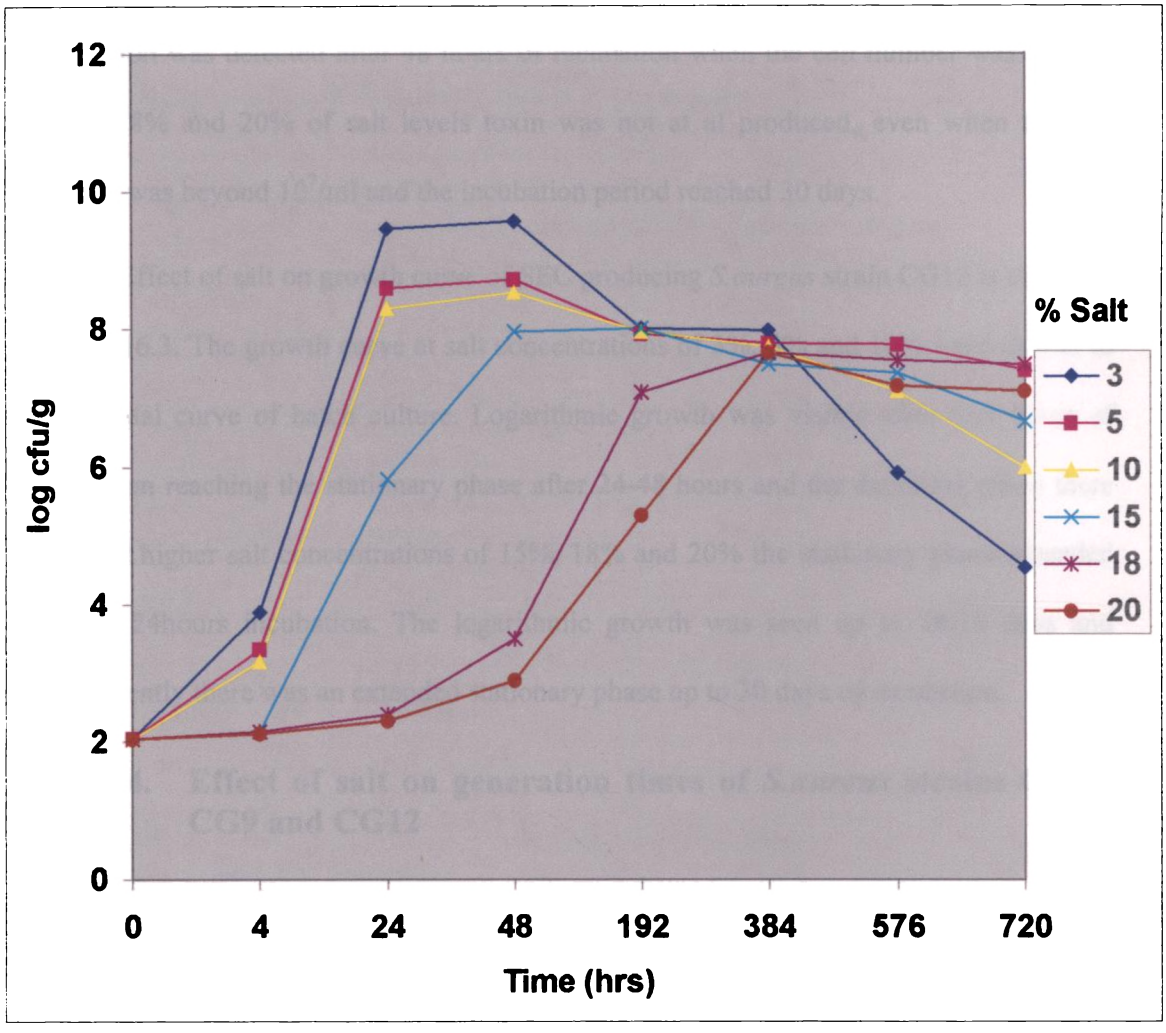


Fig.4.2.16.3. Effect of salt on growth curve of enterotoxin C producing *S. aureus* strain CG12.

Enterotoxin production was detected in 3% and 5% salt levels in 24 hours of incubation, when the respective cell population was 10^8/ml. No toxin was detected at 10% salt level, even though the cell count was 10^8/ml. But toxin was produced at 10% salt level also at detectable level after 48 hours of incubation. At 15% salt level, toxin production was detected after 48 hours of incubation when the cell number was 10^7/ml, but at 18% and 20% of salt levels toxin was not at al produced, even when the cell number was beyond 10^7/ml and the incubation period reached 30 days.

Effect of salt on growth curve of SEC producing *S.aureus* strain CG12 is shown in Fig.4.2.16.3. The growth curve at salt concentrations of 3%, 5% and 10% were similar to the normal curve of batch culture. Logarithmic growth was visible after four hours of incubation reaching the stationary phase after 24-48 hours and the declining phase there after. At higher salt concentrations of 15%, 18% and 20% the stationary phase extended beyond 24hours incubation. The logarithmic growth was seen up to 18-16 days and subsequently there was an extended stationary phase up to 30 days of incubation.

4.2.16.4. Effect of salt on generation times of *S.aureus* strains CG13, CG9 and CG12

The Table 4.2.16.4 shows the generation times of the enterotoxic *S.aureus* strains CG13, CG9 and CG12 at various NaCl concentrations in the growth media. In the case of all the three cultures, the salt concentration had a significant effect on generation time. Higher the salt concentration longer the generation time. The shortest generation times were obtained for all the three strains at 3% salt concentration, 31 minutes for SEA producing, 27 minutes for SEB producing and 39 minutes for SEC producing *S.aureus* strains. At 5% salt level the generation time for SEA producing *S.aureus* strain was 1.41 hours, and 55 minute each for SEB and SEC producing *S.aureus* strains. At 10% salt level, the generation times were above 1 hour for all the three strains. At higher concentrations of 15-20% salt, the growth times were 2.23 hours to 22 hours.

4.2.16.4. Effect of salt (NaCl) on generation time *S.aureus* strains isolated from fish and fishery products

Culture No	Salt concentration and generation time					
	3%	5%	10%	15%	18%	20%
CG13 (SEA)	31 minutes	1.41 hours	1.49 hours	2.23 hours	9.91 hours	22 hours
CG9 (SEB)	27 minutes	55 minutes	1.11 hours	1.93 hours	11.17 hours	18 hours
CG12(SEC)	39 minutes	55 minutes	1.06 hours	1.75 hours	11.42 hours	16 hours

NaCl even though a significant component in the composition of the culture media for *S.aureus* the level of the salt in the media has a noteworthy effect on the growth and toxin production. In the present study the effect of NaCl incorporated in the BHI broth were 3% - 25% of salt has been determined in the case of three selected enterotoxigenic strains namely SEA producing strain CG13, SEB producing strain CG 9 and SEC producing strain CG12. Generally a common behaviour was noticed in the case of all the three strains towards salt level. Higher the salt level, longer the generation time and delay in toxin production up to salt content of 15%. Beyond 15% of salt there was delayed growth of the organism and toxin was not at al produced by any of the *S.aureus* strains tested, except a detectable level of toxin being produced by SEA producing *S.aureus,* after 24 days of incubation at 18% salt level.

Many reports are available on the effect of salt on the growth of enterotoxigenic *S.aureus* cultures. Nunheimer and Fabian (1940) showed that the growth of enterotoxigenic strain was inhibited at 15 - 17.5% of salt. A salt level beyond 20% had a germicidal effect as well. Parfentjev and Catelli, (1964) observed that no inhibition of growth was observed at NaCl concentration of 10% or less in tryptose phosphate broth. The obsevations made in the present study are in general agreement with these findings. But Genigeorgis and Sadler (1966) reported a germicidal effect at 12% salt content when the pH of the medium was 5, while growth was supported at 16% salt content when the pH of the medium was 6.9. The studies of Mc Lean *et al* (1968) indicated that NaCl concentration of 10% had relatively little effect on the growth of *S.aureus.*

Effect of salt on toxin production has also been the subject of investigation by many authors. Riemann *et al,* (1972) found that toxin production by *S.aureus* strains were completely inhibited at 10% salt level. Mc Lean *et al,* (1968) showed that the enterotoxin production above 3% salt level was definitely affected by an increase in salt content. Markus and Silverman, (1970) found that the enterotoxins B production by *S.aureus* was inhibited by a high concentration of NaCl. The findings in the present study are in

agreement with these reports. Enterotoxin production of *S.aureus* strains were significantly affected when the salt concentration was beyond 10%. The inhibition of enterotoxin production cannot be considered as due to the effect of suppression of growth alone, because when the cell population was 10^7/ml enterotoxins was found to be produced at 3% salt level while no toxin was detected in the culture medium when the cell count was 10^8/ml when the salt level was 18% and above particularly in the case of enterotoxigenic strains producing SEA and SEC.

4.2.17. Effect of water activity (a_w) of the culture medium on growth and toxin production by enterotoxigenic *S.aureus* strains from fish and fishery products.

The water activity of the growth medium (a_w) was adjusted by the addition of appropriate concentration of NaCl in BHI broth. The 0.99 a_w corresponds to 3% NaCl, 0.98 a_w corresponds to 5% NaCl , a_w 0.945 corresponds to 10% salt, a_w 0.91 corresponds to 15% NaCl, a_w 0.87 corresponds to 18% NaCl and a_w 0.86 corresponds to 20% NaCl. The Table 4.2.17 shows the minimum time taken for production of detectable level of toxin at each water activity. The corresponding *S.aureus* population per ml is also shown in the Table. In the case of all the three enterotoxigenic *S.aureus* strains, detectable levels of toxin were produced within 24 hours when the water activity was 0.98 and above. In the case of the SEA producing *S.aureus* CG13, enterotoxin was produced in 24 hours even at a water activity of 0.945. At lower water activity of 0.91, 48 hours were required for toxin production by all the three cultures. At water activity of 0.87, enterotoxin A was produced within 16 days of incubation, but enterotoxin B were not produced at detectable level even after 30 days of incubation at a water activity of 0.87 and 0.86. At water activity of 0.86, SEA was also not produced even at 30 days of incubation. The results indicated that water activity was a critical factor in enterotoxin production by *S.aureus* strains.

4.2.17. Effect of water activity (a_w) of culture medium on growth and toxin production by enterotoxigenic *S.aureus* strains from fish and fishery products

S.aureus strain	Water activity (a_w)	Growth cfu/ml	Toxin production	Time taken for toxin production
CG – 13 (SEA producing)	0.99	$2.6x10^8$	+ve	24 hrs
	0.98	$2.55x10^8$	+ve	24 hrs
	0.945	$2.25x10^8$	+ve	24 hrs
	0.91	$9.5x10^7$	+ve	48 hrs
	0.87	$7.5x10^6$	+ve	16 days
	0.86	$1.5x10^5$	-ve	30 days*
CG-9 (SEB producing)	0.99	$9.5x10^7$	+ve	24 hrs
	0.98	$7x10^8$	+ve	24 hrs
	0.945	$5.4x10^8$	+ve	48 hrs
	0.91	$7x10^5$	-ve	30 days*
	0.87	$5x10^7$	-ve	30 days*
	0.86	$8.5x10^7$	-ve	30 days*
CG-12 (SEC producing)	0.99	$2.9x10^9$	+ve	24 hrs
	0.98	$3.9x10^8$	+ve	24 hrs
	0.945	$3.37x10^8$	+ve	48 hrs
	0.91	$9.25x10^7$	+ve	48 hrs
	0.87	$1.5x10^7$	-ve	30 days*
	0.86	$3x10^8$	-ve	30 days*

* Observations were limited to 30 days

It can be seen from Table 4.2.17 that *S.aureus* strains were capable of growth even at a water activity of 0.86. Martin *et al,* (2001) found that *S.aureus* cultures grew even at a water activity level of 0.83 which is reported to be too low for many competing microorganisms. Troller and Stinson, (1975) had observed that there was only scanty growth by *S.aureus* strains at a water activity of 0.89 and below. In another study Troller, (1972) had found that the generation time of *S.aureus* cultures were extended from 30 minutes at a water activity of 0.98 to 900 minutes for a water activity of 0.885. In the present study a similar extension of generation time to 1320 minutes have been observed (Table 4.2.16.4).

Troller and Stinson, (1975) had reported that *S.aureus* was the most drought tolerant pathogenic bacteria with a low limit of water activity of 0.86 to 0.83 in different substrates. The effect of water activity on toxin production depended on the media as well. While in media like BHI enterotoxin A was produced at a water activity of 0.90, toxin was not produced in potato media at water activity below 0.95. Potter and Leistner, (1978) found that at 30°C enterotoxin A was produced by two strains of *S.aureus* at water activities ranging between 0.864 and 0.867 within 7 days of incubation. When the temperature of incubation was decreased to 25°C, toxin production was not detected even after 2 weeks of incubation at water activities of 0.87 and 0.887. Troller, (1971) observed that in potato media, enterotoxin B was produced at a water activity of 0.93 but enterotoxins A and C could not be detected in potato media at water activity below 0.97.

The present study showed that the critical water activity at which SEA could be produced was 0.87, for enterotoxins B, a_w 0.945 and for enterotoxins C a_w 0.91.

4.2.18. Enterotoxin production of *S.aureus* strains in raw and cooked shrimp

The growth and toxin production by enterotoxigenic *S.aureus* in raw seafood was investigated at different temperatures viz; room temperature (28±2°C), 37°C, 8°C, 0°C and -20°C as described in the Materials and methods Section. The large size marine

shrimps (*Penaeus indicus*) in the ocean fresh condition were used as substrates however the raw meat had an initial native bacterial load of 10^4cfu/g and the composition of native flora was unknown. In order to simulate the native condition, this basal bacterial load was not disturbed by any treatment. Known cells of freshly cultured and saline (0.85%NaCl) washed *S.aureus* cells were inoculated at two levels viz; less than 100 and > 1000-10000 cells/g of the raw shrimp meat. The count was determined by spread plate method on B.P plate. Concurrently the enterotoxin was also determined by RPLA method. Samples stored at low temperatures namely 0°C, 8°C and -20°C. The study was continued up to 10-30 days and the resultant *S.aureus* cells were determined and the presence of toxin tested.

4.2.18.1. Effect of temperature on the growth and toxin production by SEA producing *S.aureus* strain CG13 in raw shrimp meat

Table 4.2.18.1 gives the results. In the case of the SEA producing *S.aureus* strain CG13, there was good growth at room temperature and 37°C when the inoculam was high (10^5cells/g) but toxin was not produced in 6 hours. However after 24 hours of storage toxin was produced. When the initial inoculum was very low (72/g), toxin was not produced even after 24 hours of storage. The growth of *S.aureus* also was comparatively at a slower rate. One can conclude that at low initial cell number *S.aureus* could not compete with the basal native bacterial cell already present in the raw meat and grow to attain a cell population sufficient to produce detectable level of enterotoxins. However when the initial inoculam size of *S.aureus* was very high compared with the basal bacterial load of the raw sample, *S.aureus* could establish sufficient population to produce detectable levels of enterotoxins.

In the case of storage at low temperatures, the initial inoculam of the *S.aureus* cells were kept higher 10^3- 10^4/g meat and the storage period was up to 10 days for 0°C and 8°C and 30 days for frozen storage at -20°C. It can be seen from the Table that at 0°C, *S.aureus* did not grow in raw shrimp meat and showed a decrease in count by the 10^{th} day

4.2.18.1. Effect of temperature on growth and toxin production by enterotoxin A (SEA) producing *S.aureus* strain CG13 in raw shrimp meat

No of *S.aureus* cells /g inoculated	Time	*S.aureus* count/g on B.P	Toxin production
a) Storage at room temperature (28±2 °C)			
$7.2x10^5$/g	6 hr	$1.4x10^6$	N.D
	24 hr	$4x10^7$	Toxin produced
72/g	6 hr	< 1000/g	N.D
	24 hr	$6x10^6$	N.D
b) at 37°C			
$7.2x10^5$/g	6 hr	$1.1x10^7$	N.D
	24 hr	$2.6x10^7$	Toxin produced
72/g	6 hr	<1000	N.D
	24 hr	<1000	N.D
c) at 0°C			
$4x10^3$/g	24 hr	$2x10^3$	N.D
	8 days	$2x10^3$	N.D
	10 days	$8x10^2$	N.D
d) at 8 °C			
$8x10^4$/g	24 hr	<1000	N.D
	8 days	$2x10^5$	N.D
	10 days	$5.65x10^5$	N.D
e) at –20 °C			
$9.4x10^4$/g	24 hr	$8.5x10^4$	N.D
	8 days	$7.2x10^4$	N.D
	16 days	$6x10^4$	N.D
	24 days	$5.4x10^4$	N.D
	30 days	10^4	N.D

* Raw shrimp meat carried a basal native bacterial load of $4x10^4$cfu/g

of storage. At 8°C, there was a slower growth and by 10^{th} day of storage there was a ten-fold increase in the number of *S.aureus* strains. At frozen storage temperature of -20°C, there was no growth but the viability of *S.aureus* inoculated was sustained, even after 30 days of storage. There was no toxin production in the lower temperatures.

4.2.18.2. Effect of temperature on the growth and toxin production by SEB producing *S.aureus* strain CG9 in raw shrimp meat

The growth and toxin production by SEB producing *S.aureus* strain CG9 is presented in 4.2.18.2. The inoculated raw shrimp was stored at room temperature (28±2°C), 37°C, 0°C, 8°C and -20°C and the growth and toxin production were determined at 6 hours and 24 hours intervals, in the case of room temperature and 37°C, and at 10 days of storage for 0°C and 8°C stored samples. In the case of -20°C stored samples the analysis was done upto 30 days. The basal native bacterial population was $4x10^4$cfu/g. At room temperature and 37°C there was significant growth of *S.aureus* cells within 6 hours reaching 10^5-10^6cfu/g. But toxin was not produced in detectable levels. W ithin 24 hours, the population had attained 10^9cfu/g and a toxin level above 32 microgram/g was detected in these samples. At storage temperatures of 0°C, 8°C and -20°C, there was no growth or toxin production noticed, indicating that SEB producing *S.aureus* could not grow at low temperatures and so no toxin was produced.

4.2.18.3. Effect of temperature on growth and toxin production by SEC producing *S.aureus* strain CG12 in raw shrimp meat

The results are presented in Table 4.2.18.3. The storage temperatures were the same as previous two strains and similar growth pattern with respect to storage temperature and toxin production was noticed in this case also.

4.2.18.4. Effect of temperature on growth and toxin production by enterotoxigenic *S.aureus* strains in raw fish meat

The effect of storage temperature on growth and toxin production by enterotoxigenic *S.aureus* strains in raw fish meat were studied using one SEB producing

4.2.18.2. Effect of temperature on growth and toxin production by enterotoxin B (SEB) producing *S.aureus* strain CG9 in raw shrimp meat

No of *S.aureus* cells/g inoculated	Time	*S.aureus* count /g on B.P	Toxin production
a) Storage at room temperature (28±2 °C)			
$2x10^3$/g	6 hr	$4x10^5$	N.D
	24 hr	$2x10^9$	Toxin produced
b) at 37°C			
$2x10^3$/g	6 hr	$1.5x10^6$	N.D
	24 hr	$8x10^9$	Toxin produced
c) at 0°C			
$1.5x10^3$/g	24 hr	$1.1x10^3$	N.D
	8 days	$8.4x10^2$	N.D
	10 days	$2x10^2$	N.D
d) at 8°C			
$1.5x10^3$/g	24 hr	$1.65x10^3$	N.D
	8 days	$9x10^4$	N.D
	10days	$1.25x10^5$	N.D
e) at -20°C			
$6x10^5$/g	24 hr	$1.85x10^4$	N.D
	8 days	$1.75x10^4$	N.D
	16 days	$1.45x10^4$	N.D
	24 days	$1.35x10^4$	N.D
	30 days	$1.02x10^4$	N.D

4.2.18.3. Effect of temperature on growth and toxin production by enterotoxin C (SEC) producing *S.aureus* strain CG12 in raw shrimp meat

No of *S.aureus* cells/g inoculated	Time	*S.aureus* count/g on B.P	Toxin production
a) Storage at room temperature (28±2 °C)			
$8.6x10^2$/g	6 hr	$3.5x10^6$	N.D
	24 hr	$6x10^7$	Toxin produced
b) at 37°C			
860/g	6 hr	$4.6x10^6$	N.D
	24 hr	$8.8x10^7$	Toxin produced
c) at 0°C			
$5x10^4$/g	24 hr	$5.8x10^3$	N.D
	8 days	$2x10^3$	N.D
	10 days	$1.5x10^3$	N.D
d) at 8°C			
$7.5x10^3$/g	24 hr	$5x10^3$	N.D
	8 days	$2.8x10^4$	N.D
	10days	$8x10^4$	N.D
e) at -20°C			
$1.5x10^5$/g	24 hr	$8.5x10^4$	N.D
	8 days	$6x10^4$	N.D
	16 days	$5.2x10^4$	N.D
	24 days	$4.5x10^4$	N.D
	30 days	$2x10^4$	N.D

4.2.18.4. Effect of temperature on growth and toxin production by enterotoxin B (SEB) producing *S.aureus* strain CG9 in raw fish meat

No of *S.aureus* cells/g inoculated	Time	*S.aureus* count/g on B.P	Toxin production
a) Storage at room temperature 28±2°C			
73/g	6 hr	<1000	N.D
	24 hr	<1000	N.D
742/g	6 hr	$2x10^4$cfu/g	N.D
	24 hr	$2x10^5$cfu/g	N.D
b) at 37°C			
73/g	6hr	$4x10^4$cfu/g	N.D
	24 hr	<1000	N.D
742/g	6 hr	$6x10^5$cfu/g	N.D
	24	<1000	N.D
c) at 0°C			
$8x10^3$/g	48 hrs	$1.2x10^3$cfu/g	N.D
	8 days	$2x10^2$cfu/g	N.D
	10 days	$<10^2$	N.D
d) at 8°C			
$8x10^3$/g	48 hrs	10^3cfu/g	N.D
	8 days	10^4cfu/g	N.D
	10 days	$< 10^2$	N.D
e) at -20 °C			
$8x10^4$/g	24 hrs	$2x10^4$	N.D
	8 days	$2x10^4$	N.D
	16 days	$1.6x10^4$	N.D
	24 days	$9.8x10^3$	N.D
	30 days	$9x10^3$	N.D

S.aureus strain only, because the growth behavior and enterotoxin production of enterotoxigenic strains were found to be very similar when studied in raw shrimp meat. The raw meat of seer fish (*Scomberomorous commerson*) was used for the experiment. From ocean fresh seer fish, the meat was aseptically cut and used for the study. The raw fish meat carried a basal bacterial population of 2.8x 10^3 cfu/g. In the experiments at room temperature and 37°C, two levels of inocula viz; 73 cells/g and 742cells/g were tried while for storage studies at 0°C, 8°C and -20°C, the inocula were 10^3. 10^4cells/g. The data presented in Table 4.2.18.4 shows that the growth of *S.aureus* cells in raw fish meat was not very significant. At room temperature and 37°C, there was definite growth but the presence of nasal bacterial population has significantly affected the growth of *S.aureus* at both the temperatures. The *S.aureus* population after 24 hours was not enough to produce a detectable level of enterotoxin. At storage temperatures of 0°C, 8°C and -20°C *S.aureus* has not shown any noticeable growth; instead there was a decrease in population during storage. No toxin was detected. So it is concluded that raw fish meat, having a basal native bacterial population, did not support a significant increase in population of *S.aureus* and consequently detectable levels of toxin had not been produced.

The growth of *S.aureus* in substrates like raw fish and shrimp homogenate was greately affected by the presence of other competing microflora. It has been generally agreed that *S.aureus* is a very poor competitor with other microorganisms, particularly spoilage microflora present in raw food products. As a consequence, *S.aureus* fails to attain adequate cell population to produce significant amount of enterotoxins. Noleto *et al*, (1987) has shown that enterotoxin was produced by *S.aureus* only when they could significantly outnumber the competing microorganisms growing together. Iyer and Shrivastava, (1988) had investigated the growth and survival of coagulase positive *Staphylococcus aureus* in raw shrimp and found that at frozen storage temperature, the *S.aureus* strains were found to decrease significantly; but these authors have not attempted to study the formation of toxin in fish tissue. The present study appears to be

pioneering so far as enterotoxin production by *S.aureus* in raw shrimp and fish meat are concerned.

4.2.19. Effect of temperature on growth and toxin production by enterotoxigenic *S.aureus* strains in cooked shrimp and fish meat

The effect of temperature on growth and toxin production by *S.aureus* strains from fish and fishery products were studied using cooked shrimp meat and fish meat as substrates. Known number of the *S.aureus* cells were inoculated into the cooked meat and stored at room temperature(28±2°C), 37°C, 0°C, 8°C and -20°C. The growth of the organism and toxin production were determined at regular intervals as described in Materials and Methods section.

4.2.19.1. Effect of temperature on growth and toxin production in SEA producing *S.aureus* strain in cooked shrimp meat.

Table 4.2.19.1 shows the results. At room temperature and 37°C, two levels of inoculam viz; 10 cells/g and 1000 cells/g were employed while at lower temperatures the inoculam size was 10^3-10^4cfu/g.

From the Table, it can be seen that at room temperature and 37°C, the growth of *S.aureus* in cooked shrimp meat was very rapid. In 6 hours, an initial inoculam size of 10 cells/g attained a population of $2.6x10^5$ cells/g at room temperature while at 37°C the cell number reached 10^6cells/g. However no toxin was produced after 6 hours. In 24 hours time, the *S.aureus* population at room temperature as well as 37°C reached a level above 10^7/g and detectable levels of enterotoxin were produced. When the initial inoculam size was 1000/g, in 6 hours time the cell number has reached 10^7 at room temperature but toxin was not detected. However at 37°C for the same inoculam size, the cell number had attained 10^8/g and also the toxin was produced. At lower temperatures of storage viz; 0°C, 8°C and at -20°C the cells remained just viable or even decreased on continuous storage.

4.2.19.1. Effect of temperature on growth and toxin production by enterotoxin A (SEA) producing *S.aureus* strain CG13 in cooked shrimp meat

No of *S.aureus* cells/g inoculated	Time	*S.aureus* count/g on B.P	Toxin production
a) Storage at room temperature (28±2 °C)			
10/g	6 hr	$2.6x10^5$	N.D
	24	$2x10^7$	Toxin produced
1000/g	6	$3x10^7$	N.D
	24	$1.24x10^9$	Toxin produced
b) at 37°C			
10/g	6 hr	$8x10^6$	N.D
	24	$6.6x10^7$	Toxin produced
1000/g	6	$2x10^8$	Toxin produced
	24	$1.8x10^9$	Toxin produced
c) at 0°C			
$8x10^3$/g	24 hr	$6.2x10^3$	N.D
	8 days	$4x10^3$	N.D
	10 days	$3x10^3$	N.D
d) at 8°C			
$2.3x10^3$/g	24 hr	$1.5 x10^3$	N.D
	8 days	$2.5x10^4$	N.D
	10 days	$8x10^5$	N.D
e) at -20°C			
$4x10^4$/g	24 hr	$2x10^4$	N.D
	8 days	$1.8x10^4$	N.D
	16 days	$1.6x10^4$	N.D
	24 days	$1.2x10^4$	N.D
	30 days	$8x10^3$	N.D

It is very significant to note that no enterotoxin was detected at lower temperatures of storage in cooked shrimp meat.

4.2.19.2. Effect of temperature on growth and toxin production by SEB producing *S.aureus* strain CG 9 in cooked shrimp meat

The results of the study are presented in 4.2.19.2. At room temperature and 37°C, the inoculam size was 200 cells/g while at 0°C, 8°C and -20°C the inoculam size was in the order of 10^3-10^5cfu/g. It can be noticed from the Table that SEB producing *S.aureus* strain CG9 grew well in cooked shrimp tissue both at room temperature and 37°C and produced detectable levels of toxin. This is an observation, at variance from the case of SEA producing *S.aureus* strain. However at lower temperatures of 0°C, 8°C and -20°C the SEB producing *S.aureus* strains behaved very similar to the SEA producing strain both in growth and toxigenesis. No growth or toxin production was seen at lower temperatures.

4.2.19.3. Effect of temperature on growth and toxin production by SEC producing *S.aureus* strain CG12 in cooked shrimp meat.

The data are presented in Table 4.2.19.3. The SEC producing *S.aureus* strain CG12 grew well at room temperature as well as 37°C in cooked shrimp meat, but toxin was not produced after 6 hours. Toxigenesis was apparent only after 24 hours of storage at room temperature and 37°C. At lower temperatures of 0°C, 8°C and -20°C the *S.aureus* strain did not show any growth, but on storage there was decrease in cell number. No toxin was produced at low temperatures.

4.2.19.4. Effect of temperature on growth and toxin production by SEA producing *S.aureus* strain CG13 in cooked fish meat

The results are presented in Table 4.2.19.4. It can be seen from the Table that at room temperature and 37°C, *S.aureus* showed reasonably good growth in cooked fish meat after 6 hours of storage. The initial cell number of 640/g reached 10^6/g at both the temperatures but detectable levels of toxin was not produced. But after 24 hrs the cell number attained the level of 10^8/g and toxin production was also detected at both the

4.2.19.2. Effect of temperature on growth and toxin production by enterotoxin B (SEB) producing *S.aureus* strain CG9 in cooked shrimp meat

No of *S.aureus* cells/g inoculated	Time	*S.aureus* count/g on B.P	Toxin production
a) Storage at room temperature (28±2 °C)			
200/g	6 hr	$8.2x10^6$	Toxin produced
	24 hr	$1.6x10^9$	Toxin produced
b) at 37°C			
200/g	6 hr	$5x10^7$	Toxin produced
	24 hr	$8x10^9$	Toxin produced
c) at 0°C			
$8x10^3$/g	24 hr	$6x10^3$	N.D
	8 days	$3x10^3$	N.D
	10 days	$1.1x10^2$	N.D
d) at 8°C			
$1.6x10^4$/g	24 hr	$<10^3$	N.D
	8 days	$6.8x10^4$	N.D
	10days	$1.3x10^5$	N.D
e) at -20°C			
$6x10^5$/g	24 hr	$2x10^5$	N.D
	8 days	$9.75x10^4$	N.D
	16 days	$9.7x10^4$	N.D
	24 days	$9.5x10^4$	N.D
	30 days	$8x10^4$	N.D

4.2.19.3. Effect of temperature on growth and toxin production by enterotoxin C (SEC) producing *S.aureus* strain CG12 in cooked shrimp meat

No of *S.aureus* cells/g inoculated	Time	*S.aureus* count /g on B.P	Toxin production
a) Storage at room temperature (28±2 °C)			
720/g	6 hr	$9.4x10^5$	N.D
	24 hr	$2.8x10^9$	Toxin produced
b) at 37°C			
720/g	6 hr	$8x10^7$	N.D
	24 hr	$3.68x10^9$	Toxin produced
c) at 0°C			
$5x10^3$	24 hr	$2x10^3$	N.D
	8 days	$8x10^2$	N.D
	10 days	$2x10^2$	N.D
d) at 8°C			
$5x10^3$/g	24 hr	$5.2x10^3$	N.D
	8 days	$3.5x10^4$	N.D
	10days	$8x10^4$	N.D
e) at -20°C			
$6x10^5$/g	24 hr	$2x10^5$	N.D
	8 days	$9.75x10^4$	N.D
	16 days	$9.6x10^4$	N.D
	24 days	$9.2x10^4$	N.D
	30 days	$8x10^4$	N.D

4.2.19.4. Effect of temperature on growth and toxin production by enterotoxin A (SEA) producing *S.aureus* strain CG13 in cooked fish meat

No of *S.aureus* cells/g inoculated	Time	*S.aureus* count/g on B.P	Toxin production
a) Storage at room temperature (28±2 °C)			
640/g	6 hrs	$4x10^6$	N.D
	24 hrs	$1.3x10^8$	Toxin produced
b) at 37°C			
640/g	6 hrs	$9.2x10^6$	N.D
	24 hrs	$7.6x10^8$	Toxin produced
c) at 0°C			
$3x10^5$/g	48 hrs	$9.5x10^4$	N.D
	8 days	$5x10^4$	N.D
	10 days	$2x10^3$	N.D
d) at 8°C			
$8x10^4$/g	48 hrs	$8.2x10^4$	N.D
	8 days	$6x10^5$	N.D
	10 days	$7.25x10^5$	N.D
e) at -20 °C			
$6x10^5$/g	24 hrs	$2x10^5$	N.D
	8 days	$1.85x10^5$	N.D
	16 days	$1.62x10^5$	N.D
	24 days	$1.2x10^5$	N.D
	30 days	$1.05x10^5$	N.D

temperatures. At 0°C, 8°C and -20°C growth was not apparent and on continued storage, there was slight reduction in cell number. No toxin production was noticed at low temperatures.

4.2.19.5. Effect of temperature on growth and toxin production by SEB producing *S.aureus* strain CG9 in cooked fish meat

The results are presented in Table 4.2.19.5. At room temperature and 37°C, the SEB producing *S.aureus* showed good growth in cooked fish meat but toxins were not detected. After 24 hours of storage when the cell count attained 10^8/g, detectable level of enterotoxin B was produced. At low temperatures there was no growth or toxin production in cooked fish meat by the SEB producing *S.aureus* strain.

4.2.19.6. Effect of temperature on growth and toxin production in SEC producing *S.aureus* strain CG12 in cooked fish meat

The results are presented in 4.2.19.6. At room temperature and 37°C, the inoculam size was only 94/g while at low temperatures of °C, 8°C and -20°C the inoculam size was 10^3-10^5cells/g. After 6 hours of storage at room temperature and 37°C, the SEC producing *S.aureus* strain grew rapidly and attained a population level of 10^4-10^5 but detectable level of toxin was not produced. After 24 hours of storage, the cell number reached 10^8/g in both case and detectable levels of SEC produced in cooked fish meat. At lower temperatures of storage the *S.aureus* strains did not show any significant growth but on continuous storage a slight decrease in count was noticed. No toxin was produced at low temperatures of storage.

The growth and enterotoxin production pattern in cooked shrimp as well as fish meat were quite different from what observed in the case of raw shrimp and fish meat. In the cooked shrimp or fish meat, there was no residual bacterial flora and there was no competitor for the growth of *S.aureus* strain. Further the cooked meat is a better substrate for the bacteria than the raw meat; so one could expect a faster growth and toxigenesis by *S.aureus* strains. Both cooked shrimp and fish meat supported the growth of

4.2.19.5. Effect of temperature on growth and toxin production by enterotoxin B (SEB) producing *S.aureus* strain CG9 in cooked fish meat.

No of *S.aureus* cells /g inoculated	Time	*S.aureus* count/g on B.P	Toxin production
a) Storage at room temperature (28±2 °C)			
320/g	6 hrs	$2x10^6$	N.D
	24 hrs	$1.3x10^8$	Toxin produced
b) at 37°C			
320/g	6 hrs	$4.4x10^6$	N.D
	24 hrs	$7.6x10^8$	Toxin produced
c) at 0°C			
$9x10^4$/g	48 hrs	$6x10^4$	N.D
	8 days	$5x10^2$	N.D
	10 days	$1.2x10^2$	N.D
d) at 8°C			
$9x10^4$/g	48 hrs	$8x10^4$	N.D
	8 days	$6x10^5$	N.D
	10 days	$8.5x10^5$	N.D
e) at -20 °C			
$1.5x10^5$/g	24 hrs	$9.5x10^4$	N.D
	8 days	$9.3x10^4$	N.D
	16 days	$9.2x10^4$	N.D
	24 days	$9x10^4$	N.D
	30 days	$8.6x10^4$	N.D

4.2.19.6. Effect of temperature on growth and toxin production by enterotoxin C (SEC) producing *S.aureus* strain CG 12 in cooked fish meat.

No of *S.aureus* cells/g inoculated	Time	*S.aureus* count/g on B.P	Toxin production
a) Storage at room temperature (28±2 °C)			
94/g	6 hrs	$2x10^4$	N.D
	24 hrs	$3.64x10^8$	Toxin produced
b) at 37°C			
94/g	6 hrs	$2x10^5$	N.D
	24 hrs	$7x10^8$	Toxin produced
c) at 0°C			
$9.4X10^3$/g	48 hrs	$5x10^3$	N.D
	8 days	$1.2x10^3$	N.D
	10 days	$2x10^2$	N.D
d) at 8°C			
$9.4x10^3$/g	48 hrs	$1.12x10^4$	N.D
	8 days	$6x10^4$	N.D
	10 days	$9.25x10^4$	N.D
e) at -20 °C			
$6x10^5$/g	24 hrs	$4x10^5$	N.D
	8 days	$3.5x10^5$	N.D
	16 days	$2.5x10^5$	N.D
	24 days	$1.8x10^5$	N.D
	30 days	$1.1 x10^5$	N.D

enterotoxigenic strains and they were found to produce detectable level of enterotoxin at room temperature and 37°C after a reasonable storage period. The behavior of all the enterotoxigenic strains tested was similar in both media with respect to the incubation temperature and inoculam size. An exception was the SEB producing *S.aureus* strain which quite readily produced detectable level of enterotoxin after 6 hours of incubation in cooked shrimp meat. At lower levels of storage, all the three toxigenic strains did not show any significant growth and consequently no toxin was produced in the cooked shrimp and fish media. The pattern of the effect of storage temperature on growth and toxin production of the *S.aureus* was the same as observed in the *in vitro* studies using BHI media as discussed earlier under (4.214.1- 4.2.14.4).

The literature is very scanty on the production of enterotoxins in the fish media by *S.aureus* strains. Studies of Noterman and Von Olterdijk, (1985) on *Staphylococcus* growth on pork and chicken found that 100 microgram/g of enterotoxins can be produced on prolonged incubation. Yang *et al,* (1988) has reported that cooked turkey meat is a very poor medium for enterotoxin production. In the present study, it has been found that cooked shrimp is an excellent medium for growth and enterotoxin production by *S.aureus* strains, isolated from fish and fishery products at ambient temperatures. However at low temperatures, *S.aureus* strains are not capable of growth and toxin production in cooked shrimp meat. Hence refrigerated storage of fishery products whether raw or cooked is an excellent way of preventing growth and toxin production of *S.aureus*.

4.2.20. Enterotoxin production *vis-à-vis* enterotoxic genes in strains of *S.aureus* from fish and fishery products

Enterotoxins produced by *S.aureus* are encoded by specific enterotoxic genes. The gene responsible for staphylococcal enterotoxin A (SEA) is '*sea*', gene for staphylococcal enterotoxins B (SEB) is '*seb*' , for staphylococcal enterotoxins C (SEC) is '*sec*' and for enterotoxins D is '*sed*'. A study was made on all the 25 coagulase positive *S.aureus* strains and the selected 25 coagulase negative *Staphylococcus* strains from fish and

4.2.20. Enterotoxin production and enterotoxigenic genes in *S.aureus* strains from fish and fishery products

S.aureus strains	Enterotoxin production by RPLA method				Enterotoxigenic genes by PCR method			
	Enterotoxins produced				Enterotoxigenic genes			
	SEA	SEB	SEC	SED	*sea*	*seb*	*sec*	*sed*
CG1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CG2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CG3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CG4	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CG5*	-ve	**+ve**	-ve	-ve	-ve	**+ve**	-ve	-ve
CG6	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CG7*	**+ve**	-ve	**+ve**	-ve	**+ve**	-ve	**+ve**	-ve
CG8	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CG9*	-ve	**+ve**	-ve	-ve	-ve	**+ve**	-ve	-ve
CG10*	-ve	**+ve**	-ve	-ve	-ve	**+ve**	-ve	-ve
CG11*	**+ve**	**+ve**	-ve	-ve	**+ve**	**+ve**	-ve	-ve
CG12*	-ve	-ve	**+ve**	-ve	-ve	-ve	**+ve**	-ve
CG13*	**+ve**	-ve	-ve	-ve	**+ve**	-ve	-ve	-ve
CG14*	**+ve**	**+ve**	-ve	-ve	**+ve**	**+ve**	-ve	-ve
CG15*	**+ve**	**+ve**	-ve	-ve	**+ve**	**+ve**	-ve	-ve
CG16*	-ve	-ve	**+ve**	-ve	-ve	-ve	**+ve**	-ve
CG17*	-ve	**+ve**	-ve	-ve	-ve	*+ve*	-ve	-ve
CG18*	-ve	**+ve**	-ve	-ve	-ve	**+ve**	-ve	-ve
CG19*	-ve	**+ve**	-ve	-ve	-ve	**+ve**	-ve	-ve
CG20*	-ve	-ve	**+ve**	-ve	-ve	-ve	**+ve**	-ve
CG21*	-ve	-ve	**+ve**	-ve	-ve	-ve	**+ve**	-ve
CG22*	-ve	-ve	**+ve**	-ve	-ve	-ve	**+ve**	-ve
CG23*	-ve	-ve	**+ve**	-ve	-ve	-ve	**+ve**	-ve
CG24*	-ve	**+ve**	**+ve**	-ve	-ve	**+ve**	**+ve**	-ve
CG25*	-ve	-ve	**+ve**	-ve	-ve	-ve	**+ve**	-ve

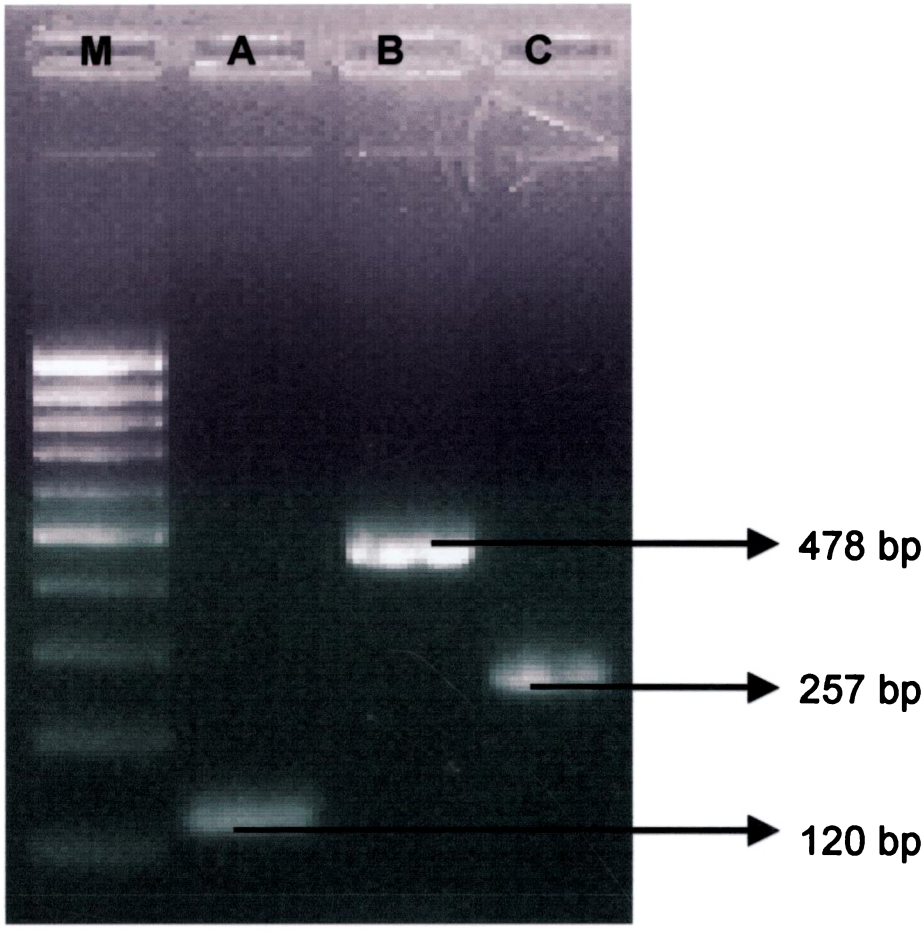
* Enterotoxigenic *S.aureus* strains

fishery products, for the presence of enterotoxigenic genes as described in Materials and Methods section.

Table 4.2.20 presents the data on enterotoxins production *vis- a-vis* with the corresponding genes in the *S.aureus* strains from fish and fishery products. The *'sea'* gene produced an amplicon in the Polymerase Chain Reaction of 127bp, *'seb'* gene an amplicon of the size of 457 bp, *'sec'* gene an amplicon of 265bp and *'sed'* gene an amplicon of 317bp Kuzma *et al,* (2003). Fig 4.2.20 gives the photograph of a typical electrophorogram of three enterotoxigenic strains of *S.aureus,* namely SEA producing strain CG13, seb producing strain CG9 and SEC producing strain CG12. The figure shows the amplicon bands corresponding to the respective toxic genes responsible for the enterotoxins produced by the *S.aureus* strains.

It can be seen from the Table 4.2.20 that out of the 25 coagulase positive *S.aureus* strains, 19 strains were enterotoxigenic. Of these 5 were SEA producing, 10 were SEB producing and 9 were SEC producing. Among them 5 produced two enterotoxins each one producing enterotoxins A and C, three producing enterotoxins A and B and one producing enterotoxins B and C. The PCR data presented in the same Table, showed that all the enterotoxin positive strains carried the corresponding enterotoxigenic genes. Single toxin producing strains had the corresponding single toxigenic genes and the multiple toxin producing strains had the corresponding genes for the multiple toxins. None of the enterotoxin negative *S.aureus* strains showed the presence of any of the toxigenic genes.

The result conclusively proved that the toxigenic *S.aureus* strains isolated from fish and fishery products had the responsible genes as well in their genome. All the *S.aureus* strains possessing the toxigenic genes could produce enterotoxins in the BHI broth in *in vitro* studies in this investigation. The study also proved that the RPLA technique (Oxoid-UK) employed in this investigation was very sensitive to detect the enterotoxins produced in the media.



M- Mol wt. Marker (100 bp), A-'sea' gene from *S. aureus*, B-'seb' gene from *S. aureus*, C-'sec' gene from *S. aureus*

Fig. 4.2.20. Agarose gel electrophoresis pattern of enterotoxigenic genes

4.2.21. Enterotoxin production *vis-a-vis* enterotoxigenic genes in coagulase negative *Staphylococcus* from fish and fishery products

The 25 coagulase negative strains comprising of *S.epidermidis*, *S.hysicus chromogens*, *S.simulans* and *S.warneri* and two untypable strains isolated from fish and fishery products were also examined for the presence of enterotoxigenic genes by polymerase chain reaction (PCR) technique. The Table 4.2.21 shows the data on the enterotoxigenic potential as well as the presence of enterotoxigenic genes. It can be seen from the Table that all of them were negative for enterotoxins production. None of them did possess any enterotoxigenic genes. The complete absence of the four enterotoxigenic genes tested in these coagulase negative *Staphylococcus* strains amply confirmed the observation that they could not produce any enterotoxins in the *in vitro* studies.

Johnson *et al,* (1991) had developed a polymerase chain reaction (PCR) protocol to detect the genes for staphylococcal enterotoxins and a set of other toxigenic genes in *S.aureus* strains from clinical specimens and contaminated foods. They had used the synthetic oligonucleotide primers targeted to internal regions of the toxin gene and the amplication fragment was detected after the PCR by agaroses gel electrophoresis. Un equivocal discrimination of toxin genes was obtained by the PCR, using nucleic acids extracted from 88 strains of *S.aureus* whose enterotoxigenicity was established biologically and immunologically. The same PCR technique was adopted in the present study also and reliable results were obtained. As shown in the Tables 4.2.20 and 4.2.21, the enterotoxins production and the presence of corresponding enterotoxic genes correlated very well. None of the isolates in the present study showed the presence of '*sed*' gene responsible for enterotoxins D. The obsevation was in ample testimony to the findings in the study that none of the 25 *S.aureus* strains tested produced enterotoxin D. Fueyo *et al,* (2005) reported that 57 out of the 269 *S.aureus* cultures from nasal carriers and manually handled foods in Spain produced at least one of the 4 enterotoxins and all these toxigenic strains carried the corresponding toxigenic genes. Scherrer *et al,* (2004)

4.2.21. Enterotoxin production and enterotoxigenic genes in coagulase negative Staphylococcus strains from fish and fishery products

Coagulase negative *Staphylococcus* strains	Enterotoxin production by RPLA method				Enterotoxigenic genes by PCR method			
	Enterotoxins				Enterotoxigenic genes			
	SEA	SEB	SEC	SED	*sea*	*seb*	*sec*	*sed*
NCG1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG4	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG5	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG6	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG7	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG8	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG9	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG10	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG11	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG12	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG13	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG14	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG15	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG16	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG17	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG18	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG19	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG20	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG21	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG22	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG23	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG24	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
NCG25	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

investigated the genotypical and toxigenic characteristics of 293 *S.aureus* isolated from milk samples and found that 42% were positive for *sec* gene corresponding to enterotoxin C (SEC), 9.6% were positive for '*sea*' gene corresponding to enterotoxins A (SEA) and 1.4% each were positive for '*seb*' gene corresponding to SEB and '*sed*' gene corresponding to enterotoxins D. They also found that the corresponding enterotoxins were produced by the *S.aureus* strains. Similar findings revealing relation between the presence of enterotoxigenic genes and enterotoxin production have been reported by Ercolini *et al,* (2004) and Nakano *et al,* (2004). The findings in the present study are in full agreement with the above reports discussed.

SUMMARY

5. SUMMARY AND CONCLUSIONS

This thesis is mainly concerned with the study of the incidence of entero toxigenic *Staphylococcus aureus* in various fish and fishery products. The *Staphylococcus* especially *S.aureus* are considered as important toxigens which can cause food poisoning and hence they are taken for the study along with selected number of coagulase negative *Staphylococcus species* giving typical colonies as that of *S.aureus* on B.Pagar plates.

The topics of the study included the investigation on total microbial quality analysis of different fresh fish and shellfish, frozen fish and shellfish, dried fish and shellfish and value added fishery products, a detailed investigation on the incidence of *Staphylococcus aureus* in different fishery products, their biochemical characterization, pathogenicity, toxigenicity, factors effecting growth and toxin production in *in-vitro* systems and in raw and cooked shrimp and fish meat, and PCR analysis of the *Staphylococcus* genomes for the toxigenic genes vis-à-vis the enterotoxigenic potential of the strains. The findings are briefly summarised as follows.

5.1. Bacterial quality of fish and fishery products

Bacterial quality of different fresh fish and shellfish, frozen fish and shellfish, dried fish and selfish and value added fishery products from fish internal trade and fish processing establishment were studied in detail. The various bacteriological parameters studied include total plate count (TPC), total enterobacteriaceae count (TEC), total coliforms, faecal coliforms, faecal streptococci, *E.coli*, *S.aureus* and pathogens like *V.cholerae*, *V.parahaemolyticus* and *Salmonella.*

In general the total microbial quality of various fish and fishery products examined were found to be unsatisfactory from the food safety point of view. Most of the samples carried the bacterial loads above the approved standards for different fish and fishery products, stipulated by USFDA, EU and BIS.

The total plate counts of fresh finfish samples were in the range of 10^5-10^6 cfu/g. Most of the samples had the total Enterobacteriaceae counts in the range 10^2-10^3 cfu/g. Total coliforms and faecal coliforms were detected in the range 20-140$^+$. Most of the faecal coliforms were *E.coli*. Faecal streptococci were detected in the range of 10^2-10^4 cfu/g. pathogens like *Salmonella* was detected in 15% and *V.choleraee* non-O1 in 25.5% of the samples. However all the samples were free from *V.parahaemolyticus*.

The microbial quality of 18 shrimp samples from the local markets was analyzed. The total plate counts of most of the samples were above 10^6cfu/g. The total Enterobacteriaceae counts were in the range 10^2-10^3 cfu/g. Total coliforms and faecal coliforms were in the range 110-140$^+$ and a significant number of coliforms were *E.coli*. *S.aureus* was detected only in two samples. Similar was the case with *Salmonella*. *V.cholerae* non-O1 was detected in 4 samples and *V.parahaemolyticus* in 5 samples.

The results of bacterial quality analysis of fresh whole crab showed a bacterial load in the range of 10^5cfu/g-10^7 cfu/g. The total Enterobacteriaceae count was in the range 10^2-10^3 cfu/g in all the samples. The coliforms were in the range 110-140$^+$. Faecal streptococci were present in all the samples in the order 10^2-10^4cfu/g. Pathogens like *V.cholerae* non-O1 was detected in one sample, while *V.parahaemolyticus* was detected in 4 samples. The critical pathogen *Salmonella* was not detected from any of the samples analyzed.

Total bacterial counts of fresh cephalopods were in the range of 10^5-10^6cfu/g for whole squid and 10^6-10^7 cfu/g for whole cuttle fish. Total coliforms and faecal coliforms were detected in most of the samples. *S.aureus* was present in 50% of the samples. *V.cholerae* non-O1 was detected only in one sample. The pathogens, *V.parahaemolyticus* and *Salmonella,* were not detected in any of the samples.

Fresh green mussel samples collected from local markets and farms of Calicut were investigated for total microbial quality. The total plate counts of farmed samples were in the range of 10^4-10^5/g, while that of samples from local markets had bacterial

counts in the range 10^6. 10^7cfu/g. The total Enterobacteriaceae counts in farmed samples were in the range of 10^2-10^4/g. Coliforms and *E.coli* counts were high in wild samples. *S.aureus* was not detected in any of the samples. *V.cholerae* non-O1 was found in 4 out of the 18 samples, *V.parahaemolyticus* in 5 samples and *Salmonella* in 3 samples.

The fresh, boiled and shucked clam meat collected from the local markets of Cochin was investigated for microbial quality parameters. Except one sample, the total plate counts of the raw samples were in the range of 10^4-10^6cfu/g, while the shucked clam meat had a total bacterial load in the range 10^6-10^8cfu/g. Total Enterobacteriaceae were more or less in the range of 10^2-10^3cfu/g. Total coliforms and faecal coliforms were mostly in the range 10^2-10^3cfu/g. Coliforms were detected in the range 110-140^+. *E.coli* was recorded in a low level only. *S.aureus* was found in 4 out of the 20 samples. *Salmonella* was detected in 2 samples. *V.cholerae* non-O1 was detected in 7 out of the 20 samples while only 4 samples carried *V.parahaemolyticus*.

Edible oysters were collected from a farm of Kollam and investigated for total microbial quality including pathogens. The total plate counts of most of the samples were in the range 10^4-10^5cfu/g. Total Enterobacteriaceae count was in the range of 10^2-10^4. Coliforms were comparatively less. Faecal streptococci were in the range 10^2-10^3 cfu/g. *S.aureus* was present in only two samples. All the samples were free from pathogens like *V.cholerae, V.parahaemolyticus* and *Salmonella.*

Microbial qualities of frozen shrimps from seafood processing plants and finfish and shrimp samples from local cold storages were analyzed for total microbial quality including pathogens. Most of the samples from local cold storages were poor in quality. The pathogens were detected from a certain percentage of the frozen fish and shellfish samples from local cold storages. The bacterial qualities of frozen shrimps meant for export were found to be good in quality. In 91% of the samples, total plate count was within the permitted 5lakh/g limit. The pathogens like *Salmonella* and *V.parahaemolyticus*

were not at all detected from any of the samples while *V.cholerae* non-O1 was detected only from one sample.

The bacterial quality analysis of dried fish showed that in most of the samples the total plate count varied between 10^3-10^5 cfu/g. The presence of *E.coli* was confirmed only in two samples. The total plate counts in dried shrimps were found to be quite high and they were in the range of 10^5-10^7cfu/g. However the pathogenic bacteria like *V.cholerae*, *V.parahaemolyticus* and *Salmonella* were not detected from any of the samples.

The microbial quality parameters of most of the value added fishery products were in acceptable limit. The presence of *S.aureus* was confirmed in 66% of the samples, however the presence of pathogens was not confirmed from any of the samples.

In general it can be concluded that both fresh and processed fish and shellfish samples sold in the local markets of Cochin were microbiologically unfit for human consumption.

5.2. Incidence of *S.aureus* in fish and fishery products

The investigation on the incidence of *S.aureus* in different fish and fishery products showed that *Staphylococcus* especially *S.aureus* was a major contaminant in most of the products. They were invariably present in most of the samples analyzed viz; in fresh fish (21.4%), fresh shrimp (11.1%), fresh crab (20%), fresh cephalopod (50%), boiled clam meat (22%), dried fish (25%), dried shrimp (8.3%), frozen shrimp (52%), frozen fin fish (50%) and value added fishery products (66%). The study showed that the incidence of *S.aureus* was high in foods, which were subjected to more human handling, and this indicated the unhygienic status of food handlers.

5.3. Studies on extracellular enzmes and biochemical characteristics associated with *S.aureus* and coagulase negative *Staphylococcus* species

Extensive studies were carried out to asses the potential of *S.aureus* to produce extracellular enzymes like lipase, phospholipase, thermonuclease, protease and phosphatase which are associated with the virulence potential of *Staphylococcus*. All the

Staphylococcus aureus isolates produced lipase, phospholipase, phosphatase and protease. Except one strain, all the others produced thermonuclease as well. Haemolysins were produced by 64% of the isolates only.

The coagulase negative *Staphylococcus* isolates showed a similar trend in the production of phospholipase, lipase and protease. But all the cultures were negative for thermonuclease. Only 40% of the isolates produced phosphatase and 44% of the isolates produced haemolysins.

Studies on the utilization of sugars and amino acids showed that among the three pentoses (arabinose, ribose and xylose), *S.aureus* could utilize only ribose with the production of acid. All the five hexoses (fructose, galactose, glucose, manose and mannitol) tested were utilized. Among disaccharides (D-cellobiose, lactose, maltose, sucrose and trehalose), except D-cellobiose all other sugars were utilized by all the isolates. None of the trisaccharides (melezitose, raffinose and salicin) were utilized by *S.aureus*.

Variations were observed in the utilization of sugars like pentoses, hexoses , disaccharides, trisaccharides and sugar derivatives among different species of coagulase negative *Staphylococcus* isolates.

The aminoacid utilization studies with four amino acids (arginine, histidine, lysine and ornithine) showed arginine as the only amino acid that could be utilized by both *S.aureus* strains and coagulase negative *Staphylococcus* strains.

5.4. Antibiotic sensitivity of *S.aureus* and coagulase negative *Staphylococcus* species

Studies were carried out on antibiotic sensitivity of the *Staphylococcus* cultures against 12 most common antibiotics. It was found that most of the strains developed multiple drug resistance towards commonly used antibiotics but they were sensitive to Methicillin, Novobiocin and Vancomycin. This study indicates the spread of drug resistant strains in the environment.

5.5. Enterotoxin production by *S.aureus* strains and coagulase negative *Staphylococcus* cultures

The production of enterotoxins by *S.aureus* and coagulase negative Staphylococci were studied in detail with the help of RPLA test kit (Oxoid, U.K). Among the 25 *S.aureus* cultures studied, 76% of the isolates were toxigenic. 20% of the isolates produced enterotoxins A, 40% produced enterotoxins B, 36% produced enterotoxins C and none of the isolates produced enterotoxins D. None of the 25 coagulase negative *Staphylococcus* isolates could produce enterotoxins. It is evident from the study that most of the *S.aureus* isolates from fish and fishery products are capable of producing enterotoxins.

5.6. Studies on growth –kinetics of *S.aureus* strains in *in-vitro* systems

Effect of environmental factors like temperature, pH, salt concentration and water activity on growth and toxin production of selected number of enterotoxigenic *S.aureus* strains were studied.

The study on effect of temperature on growth, toxin production and generation time showed that the organism was capable of growing fast and producing toxin at temperatures of 28±2°C, 37°C and 45°C but growth was rather slow at low temperatures of 8°C. Slow death was observed at 0°C and -20°C.

pH tolerance of the cultures were tested in BHI broth with pH 3, 5, 7, 9 and 12. Except enterotoxin A, all the other enterotoxins viz; SEB and SEC were produced at pH 5,7 and 9 but none of the isolates could grow and produce toxin at pH 3 and pH 12. The generation time at pH 5 and 9 were rather longer comparing with the generation time at pH 7.

Salt tolerances of cultures were tested at different salt concentrations viz: 3%, 5%, 10%, 15%, 18% and 20%. All the 3 strains of *S.aureus* tested could grow up to 20% salt level, but they showed strain-to-strain variation in toxin production. Enterotoxin A was produced up to a salt level of 18%, enterotoxins B up to 10% and enterotoxins C up to

15% salt. A direct relation ship was observed between salt concentration and generation time. Higher the salt concentration, longer the generation time and *vice-versa.*

The study on the effect of water activity on growth and generation time showed that all the three *S.aureus* strains could grow up to a water activity of 0.86. Strain to strain variation was observed in enterotoxins production. Enterotoxin A(SEA) was produced up to a water activity of 0.87, enterotoxins B (SEB) was produced up to a_w of 0.945 and enterotoxins C(SEC) up to a_w 0.91.

5.7. Studies on the effect of temperature on growth and toxin production of *S.aureus* in shrimp and fish meat.

The effect of temperature on growth and toxin production by *S.aureus* in raw and cooked fish and shrimp meat stored at different temperatures, viz; 0°C, 8°C, 28±2°C, 37°C and -20°C was studied. In raw shrimp, SEA producing strain could produce toxin after 24 hours when the initial inoculum was high, but at low inoculum level, no growth and toxin production was observed after 24 hours of incubation. At low temperatures of 0°C and - 20°C, no growth was observed and at 8°C slow growth was observed. Similar was the results with SEB and SEC producing *S.aureus* strains. Similar studies in raw fish meat with enterotoxins producing strain CG9 showed that raw fish meat did not support the growth and toxin production.

The growth studies of *S.aureus* in cooked shrimp and fish meat showed that cooked meat supported growth and toxin production after a certain period of incubation. In the case of SEB producing strain CG9, toxin was produced after 6 hours of incubation in cooked shrimp meat. At low temperatures, no growth and toxin production was observed.

It was concluded from the study that shrimp and fish, both raw and cooked when stored at refrigerated temperatures did not support the growth and toxigenesis by *S.aureus.*

5.8. Detection of enterotoxigenic genes of *Staphylococcus* strains by PCR method

The enterotoxins production potential of both *S.aureus* and coagulase negative Staphylococci were determined by PCR method by amplifying toxigenic genes. The results were compared with RPLA test results for toxin production. A good correlation was observed between PCR method and RPLA method. All the enterotoxigenic *S.aureus* strains possessed the genes responsible for enterotoxin production. None of the coagulase negative *Staphylococcus* carried any toxigenic genes. The study proved that RPLA technique (Oxoid-UK) employed in this investigation was very sensitive to detect the enterotoxins produced in the media.

Suggestions for further research

1. Fate of the *Staphylococcus* enterotoxins pre-formed in the raw fresh fish and shellfish during processing and storage.
2. Development of methods to remove/ destroy the preformed enterotoxin in fish/fishery products, without affecting the quality/saleability of processed fish/shellfish.

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List of Publications by the author

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Papers presented in symposia

1. Incidence of *Vibrio cholerae* non-O1 in seafood from the retail outlets and processing plants of Cochin area. (2005). **Sindhu O.K & P K. Surendran.** Scientific paper presented in the international symposium " *Improved sustainability of fish production systems and appropriate technologies for utilization*". Held on 16.03.05 to 18.03.05. School of Industrial Fisheries CUSAT.

2. Microbial quality and food safety of fresh and dried fish and shellfish from the retail markets of Cochin. (2005). **Sindhu O.K & P K.Surendran**. Scientific paper presented in *XV Swadesi Science Congress* held at Govt. Brennen College, Tellicherry, Kannur. Kerala. 5.11.05 - 7.11.05.

3. Incidence and antimicrobial susceptibility of *Staphylococcus* species isolated from fish and fishery products. (2005). **Sindhu O.K & P K. Surendran**. Scientific paper presented in 7^{th} *Indian Fisheries Forum* held at Bangalore from 08.11.05-12.11.05.

Papers Published in Scientific Journals

4. Entero toxigenicity of coagulase positive and negative staphylococci isolated from fish and fishery products. (2006). **Sindhu O.K & P.K.Surendran**. Fishery Technology 43(2).

5. Virulence factors associated with *Staphylococcus* species from fish and fishery products. (2007). **Sindhu O.K & P.K.Surendran.** (Communicated Fishery Technology).