Isolation and Identification of Heterotrophic and Enteric Bacteria from Fresh and Salted Hilsa (*Tenualosa ilisha*)

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TO

MY BELOVED PARENTS

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ABSTRACT

Hilsha (*Tenualosa ilisha*) is a popular, tasty fish found in the rivers of Bangladesh round the year. In our local market both fresh and salted hilsha are available. Samples were collected from different local markets of Dhaka city to investigate the microorganisms associated with fresh and salted hilsa fish. Along with the associated microorganisms nutritional quality was also studied. During this study four different market of Dhaka city *viz.* Palashi Bazaar, Karwan Bazaar, Anando Bazaar and New Market were selected for sampling.

The temperature and pH of fresh fish samples ranged from 28.6 to 32.4^{0}C and 6.55 to 6.72 respectively while the temperature and pH of salted fish samples ranged from 28.2 to 32.5ºC and 5.31 to 5.70, respectively.

Five types of bacteriological culture media *viz*. nutrient agar (a generalized complex medium), EMB agar medium for coliform, SS agar for *Salmonella-Shigella*, MSA agar for *Staphylococcus* and TCBS for *Vibrio* were used to assess the quantitative and qualitative study of bacteria associated with the hilsa fish. The bacterial load of fresh hilsa fish was ranged between 6.6×10^4 cfu/gm to 1.22×10^6 cfu/g, 1.5×10^2 to 6.35×10^3 cfu/g, 8.5×10^3 to 1.2×10^6 cfu/g and 2.5×10^2 to 1.64×10^5 on nutrient agar, SS agar, EMB agar and MSA agar respectively. On the other hand the bacterial load of salted fish samples was found to be ranged between 1.4×10^4 to 7.45×10^6 cfu/g, 6.4×10^3 to 7.75×10^4 cfu/g, 1.5×10^2 to 4×10^4 cfu/g on nutrient agar, EMB agar, and MSA agar, respectively. No bacterial colony was found on SS agar and TCBS agar plate in salted fish. The results clearly showed that fresh fish samples were associated with high bacterial loads than that of salted fish.

The highest heterotrophic bacterial count 1.22×10^6 cfu/g recorded in the fresh fish sample of Karwan Bazar. Maximum coliform bacterial count was also noticed in the fresh fish sample of same market.

During this investigation 66 bacterial colonies were isolated. Of them 24 bacterial isolates were selected for detail study. Out of 26 isolates 12 were Gram positive bacteria and 12 were Gram negative bacteria. Among the Gram positive 3 were rod shaped and two were spore former and members of the genus *Bacillus*. The non spore former bacterium was the member of *Listeria monocytogenus*. The provisionally identified *Bacillus* spp. were *Bacillus megaterium* and *B. marinus*.

The remaining 9 Gram positive bacteria were the members of *Planococcus citreus* (1), *Micrococcus variens* (1), *Micrococcus nishinomiyaensis* (1), *Staphylococcus lentus*(1), *Staphylococcus epidermidis* (2) and *Staphylococcus aureus* (3). Twelve Gram negative bacterial isolates belonged to the member of *Salmonella paratyphi* (2), *Salmonella spp.* (2), *Escherichia coli* (1), *Proteus morganii* (3), *Proteus vulgaris* (2), *Vibrio sp*.(1) and *Halobacterium* (1).

Proximate composition of raw *Tenualosa ilisha* in dorsal and ventral part was 55.47% and 46.03% moisture, 18.94% and 24.25% protein, 23.47% and 24.24% lipid and 1.64% and 2.92% ash, respectively.

In salted *T. ilisha*, the proximate composition of dorsal and ventral part was 44.88% and 40.05% moisture, 20.79% and 20.19% protein, 15.89% and 20.83% lipid and 18.15 and 18.35% ash. It was found that the protein, lipid, moisture contents decreased and ash content increased after salting condition.

CONTENTS

List of Tables

List of Figures

List of Plates

List of Symbols and Abbreviations

Symbols	Details
APC	Aerobic plate count
ATCC	American type culture collection
BCAS	Bangladesh centre for advanced studies
BGLB	Brilliant Green Lactose Bile Broth
CDC	Centre for disease control
Cfu	Colony forming unit
Cm	Centimeter
Eg	For example
et al	And others (et alliori)
EC	European commission
EU	European Union
EMB	Eosin Methylene Blue
GDP	Gross domestic product
GIT	Gastrointestinal tract
GMP	Good manufacturing practice
h	Hour
HACCP	Hazard Analysis Critical Control Point
Kg	Kilogram
L	Litre
LT	Heat labile toxin
MI	Millilitre
M	Molar
Mg	Milligram

Symbols	**Details**
Mm	Millimeter
µg	Microgram
µl	Microlitre
NS	Normal Saline
EMB	Eosine methylene blue agar
MSA	Manitol Salt Agar
TCBS	Thiosulphate Citrate Bile Sucrose agar
TBC	Total Bacterial Count
XLD	Xylose Lysine Deoxycholate agar
V.	Vibrio
E.	Escherichia
^0C	Degree centigrade
P^H	Negative logarithm of hydrogen ion concentration
NaOH	Sodium hydroxide
NaCl	Sodium Chloride
H_2S	Hydrogen sulphide
Spp	Species
TCBS	Thiosulphate citrate bile salt
UV	Ultre violate
v/v	Volume/volume
w/v	Weight/volume
(-)ve	Negative
(+)ve	Positive
RAE	Retinol Activity Equivalents

1. INTRODUCTION

1.1 Background:

Bangladesh is a south Asian country located in between latitude 20º 34´ and 26º 39´ north and longitude 80º 00´ and 92º 41´ east. It is an agro based country enriched with enormous fisheries resources. Bangladesh is blessed with huge open water resources with a wide range of variations in nature. The total inland area at present is 47,03,658 hectare (DoF 2012) that has a high potential for fisheries production. And these fishes have a great significance in the life of mankind, being a most important and cheap source of protein and providing certain useful products. Fish is a part of our cultural heritage. There is a Bengali term- "Mache Bhate Bangali". It is a well proven term as we do not think our meal without fish even once in our meal. Hence, fish is next to rice in Bangladesh.

Fishes are valuable sources of high quality protein and other organic products (McCance Rand Widdowson 1960). Fishes occupy significant position in the socio economic fabric of the South Asian countries by providing the population not only the nutrition but also income and employment opportunities (Rubbi *et al*. 2012). Basically fish take a prominent place in the mind of many people as a source of protein compared to other protein sources. Fish is considered as one of the most perishable of all food stuffs.

Fish is very important food stuff in developing countries due to its high protein content and nutritional value. Fish provides more than 50% of the animal protein for the populations of 34 countries (Bhuiyan 1987).

Among the fishes Hilsa is rich in protein, fat, vitamins and minerals. lt is considered as the national fish due to its popularity, economic importance and historically securing the largest share of landing with approximately 190 metric tons annually combined from island and marine capture (Mia and Shafi 1996). In other side five type of Hilsa can be found worldwide. Yearly Hilsa are caught 5000,000 ton. Among them 50%-60% are caught by Bangladesh, 15%-20% are caught by India, Pakistan and rest 5%-10% are caught by Malaysia, Thailand, China, Vietnam and Srilanka. Hilsa is an anadromous Clupeidae fish

occurs in the estuaries, brackish water lakes and fresh water rivers of the western division of the Indo Pacific region. It grows and lives in sea and upstream to the rivers for breeding purpose. Hilsa, the national fish of Bangladesh has been playing a very important role in our economy. Annual Hilsa production is 2 lacks 90 thousands metric tons (2007-2008). Hilsa contributes about 30% in the total fish production of the country. We earn Tk. 7000 core from Hilsa fish export annually. About 40% fisherman or 2% of total population of the country earn their livelihood depending on Hilsa fishery directly or indirectly. It contributes in national GDP about 1% (DoF 2009). Indian shad *Tenualosa ilish*a constitutes the largest single fishery of Bangladesh. Its annual catch accounts for nearly 40% of the total inland harvest of the country (Uddin M *et al*. 1998).

The Hilsa shad is largely an *anadromous* species, but two other ecotypes a fluvial *potamodromous* type and a marine type have been recognized. The *potamodromous* stocks appear to remain in the middle reaches of the rivers throughout the year and breed therein. The *anadromous* stocks, whose normal habitat is the lower region of the estuaries and the foreshore areas, ascend the rivers during the breeding season and return to the original habitat after spawning. The upstream migration during the main breeding season depends largely on the commencement of the south-west monsoon and consequent flooding of the major rivers of Bangladesh, Burma and India. Hilsa is valuable source of macro and micronutrients and play an important role to provide essential nutrients for the people of Bangladesh. River shad (*Tenualosa ilisha)* is one of the members of the genus *Tenualosa* of the family Clupeidae, order Clupeiformes. Locally known as Ilish, it has been designated as the national fish of Bangladesh. This fish is highly tasty and very much well known to the people of Bangladesh. It is rich in protein and poly unsaturated fatty acids. Its liver contains considerable amount of vitamin A, while its body oil contains vitamin C (Bhuiyan 1984). It also contains calcium, phosphorus and other mineral salts. It is estimated that one pound of *Tenualosa ilisha* fish has an average 300-1100 calories energy (Rahman 1976).

Biochemical composition of fish flesh may vary within the same species of fish depending upon the fishing season, age, sex and habitat (Srivastava CBL 1985). The variation is also found within the different region of the body (Jacquot 1961). In fishes, proximate

composition means the composition of the fish flesh. Fish flesh contains four basic ingredients in varying proportions major nutrients such as water (70-80%), protein (18-20%), fat (5%) and minerals (5%) and minor nutrients such as vitamin, carbohydrate. It has high nutritional value in terms of fats and proteins that are not commonly available in other foods. But fish is considered as one of the most perishable of all food stuffs. As soon as a captured fish dies, it begins to deteriorate. The deterioration of the flesh of the fish is caused by the action of enzymes, by micro-organism and by chemical action. Bacteria on the surface of fish skin, gills and in the guts are generally harmless in the living fish, but they start their destructive activities as soon as this fish dies. They grow and multiply rapidly at ordinary temperature, invade the flesh through the skin and breakdown the complex chemical construction of the flesh, producing the stale and later the putrid smells and tastes which are usually associated with spoilage of fish (Jadhev and Magar 1970). The activity of enzymes, bacteria and chemicals could be minimized by standards of cleanliness, careful handling technique, preservation, quality control and temperature reduction. The qualities of frozen fish is determined mainly by the total number of bacteria present and by the individual count of bacteria of public health significance such as Escherichia coli, fecal coli, coagulate positive *Staphylococcus*, *Vibrio cholerae*, *Salmonella* etc. The quality of fish and fishery products depend on various factors i.e. the freshness of the raw fish, method of handling and processing factories, pre and post process temperature etc. Strict control of every stage of processing is necessary to prevent bacterial multiplication and the various chemical changes. A regular assessment of the quality of raw material is essential especially in view of the variation in the freshness of raw materials like *T. ilisha*, where the rate of spoilage may be high and will depend on the size and species (Rahman 1976).

Preservation of fish by salt is an old age technology. This method of preservation still enjoys popularity in many developing countries owing to its simplicity and low cost of processing. When fatty fishes are salted, there is usually a certain degree of fermentation involved (Takagi *et al.* 1984). Lona ilish or salted ilish is a salt fermented product prepared from the Indian shad (*Tenualosa ilisha*, Ham-Buch 1822). Lona ilish is a very popular product and is widely consumed in Bangladesh mainly due to its typical flavor, aroma and texture. The product is sliced Hilsa, about 1.5 to 2.00 cm in thickness. The texture remains firm and the

flesh does not easily separate from its bone. It has characteristic strong aroma mixed with some sweet, fruity acidic notes along with some saltiness. The strong odor permeates the air during storage and gives the area a characteristic of lona ilish. The technology of lona ilish actually originated in Bangladesh about 100 yrs ago on the bank of river Padma and Meghna under Noakhali district. It is assumed that the technology evolved during the glut period when there were no such preservation techniques except sun drying and salting was available. Sun drying was not suitable for Hilsa like high fatty fishes due to rapid development of rancidity on being exposed to the sun. In addition, sun drying was difficult during continuous spell of rain in July-August that corresponds to the main glut period. This way of processing might have been started to quickly preserve large quantity of fish in an inexpensive way. Though the spoilage of salted fish is less severe but it is usually contaminated with the harmful bacteria like *Bacillus spp., Staphylococcus spp., Micrococcus varians, Micrococcus luteus, Pseudomonas mallei,* and the fungi like *Aspergillus niger, Aspergillus flavus and Rhizopus spp.*

Hilsa is a dark-fleshed high lipid species. Icing is an effective short-term preservation method for the fish. Sun-drying cannot be performed for the species because of atmospheric oxidation or rancidity problems. Long term chilling and freezing are not useful due to texture degradation for spoilage of dark muscles. Considering the compositional characteristics of the species, comparative advantage and acceptability of different fish preservation methods and socio-economic conditions and food habit of the local consumers, salting seems to be the best suited method for the preservation of hilsa. The following problem, however, have been found to be associated with the process and the products (Nowsad 2010):

i. The producers do not follow the regulations regarding public health and sanitation.

ii. In glut period, the fish only those are spoiled or partially spoiled and cannot be sold in the fresh wet fish market are used for salting.

iii. The fish or the cut pieces are not washed before salting in most of the cases.

iv. The raw material is contaminated by pathogens or other bacteria during scaling, gutting, dressing and cutting by unclean knife, container or tools.

v. Low quality solar-salt is used that inhibits the development of good texture, attractive colour and nice flavour of the product.

vi. Salt: fish ratio is not properly maintained. So rancidity occurs in fish during dry salting.

vii. Sometimes excess salting may denature protein and impact upon the sensory and biochemical properties of the final product.

viii. In wet salting, cut pieces are often floated on the surface of the brine, come in contact of air and become rancid.

ix. Semi-fermented ilish is not always well protected in the underground hold. Rain water and mud enter and insects and rodents attack and spoil or contaminate the products.

x. Packaging and storage are not appropriate and hygienic. Very often rancid off flavor develops in the products those are kept in the basket for long time.

Although frozen and or salted ilish is being exported in large scale there is lot of information available from Bangladesh export promotion bureau that, the exported fish and fishery products are sometimes rejected by the foreign countries due to high load of bacteria and presence of undesired pathogenic type of microorganism. Further, several ingredients are now added to seafood as additives, antioxidants, preservatives, emulsifiers, cryoprotectants and coloring materials. There are also problems of pesticide residues, toxic metals, mycotoxins, biotoxins, antibiotic residues etc. Under these circumstances, the responsibility of the processor has become increasingly complex and hence, there is a global shift from food quality to food safety. In this context, the present study was undertaken to estimate total bacterial load and specific pathogen of frozen and salted Hilsa (*T. ilisha)* and to compare their microbiological load at different market in Dhaka City and also to compare their nutritional quality.

Lona ilish is traditionally prepared by dry salting the diagonally cut Hilsa chunks followed by fermentation in saturated brine (previously boiled and cooled) in metal container till appearance of the characteristic flavor and texture. Traditional methods of preparation of

lona ilish and evaluation of some of its important biochemical and microbiological quality have given bellow.

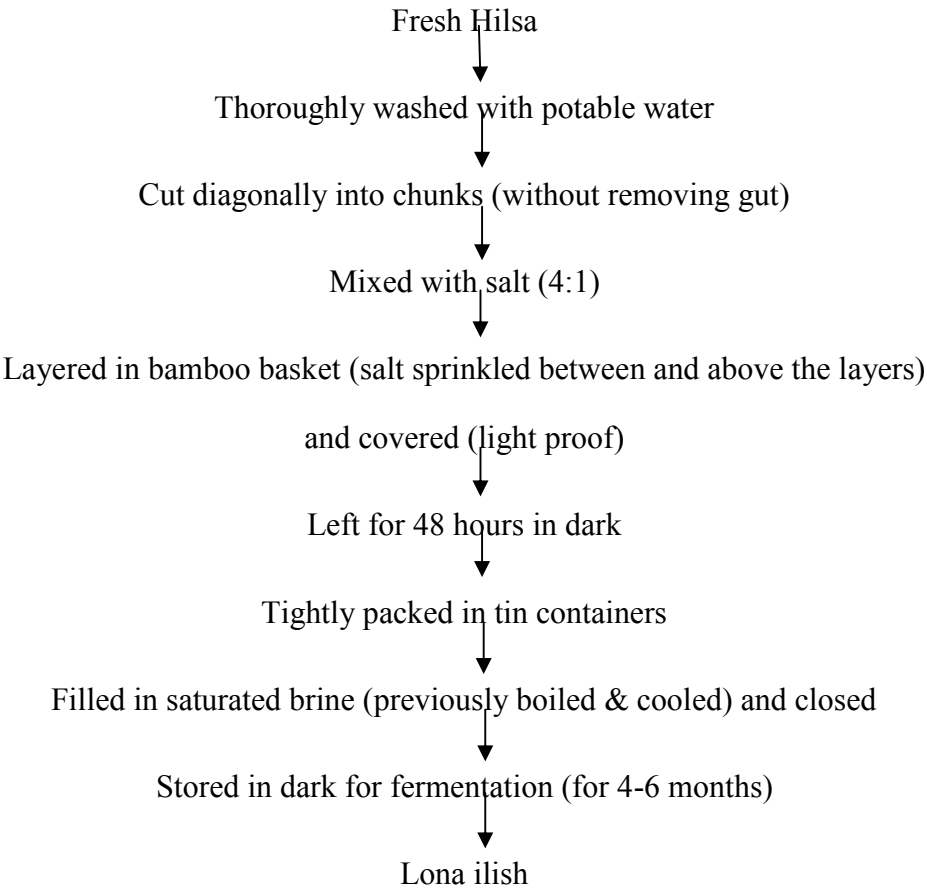


Fig.- Preparation of Lona ilish by traditional method

Fisheries plays an important role in the socio-economic development, nutrition, employment and poverty alleviation of large number of population and foreign exchange earnings in the economy of Bangladesh. It has been estimated that about 1.28 million people are directly engaged with fisheries activities and fish farmers in Bangladesh are about 3.08 million. Another 12 million people indirectly earn their livelihoods from fisheries related activities.

Hilsa was once abundant in rivers, estuaries and marine area in Bangladesh. They were usually caught by a large number of subsistent fishermen. There are some information on the sensory, biochemical and bacteriological studies of hilsa (Mansur MA *et al.* 1998). The biochemical and nutritional studies of some fresh water fish species mentioned proximate

composition of some commercial species of fresh water fish. Naser *et al* stated the proximate composition of shellfish (prawn and shrimp) in Bangladesh. Stansby has established that information on the chemical composition of fish in respect to the nutritive value is important to compare with other source of animal protein, meat and poultry products. There are a few reports on the nutritive values of Hilsa. Hilsa is a migratory fish and it travels thousands of kilometers from upstream of river to mid ocean. A different region of the Bay of Bengal contains different types of food (phyto and zooplankton). Different types of food make proximate composition a bit different and also different part of the body makes the composition different as well.

1.2 Rationale

Fish are highly perishable food item for which they start to spoil as soon as they are harvested (Balachandran 2001). So processing and storage method of fisheries product is a vital factor in fish consumption. On the way of transportation from each point to market, there is a great chance for the fish to be contaminated by bacteria (Clucas and Ward 1996). *Salmonella*, *Shigella*, coliform and other bacteria are considered in terms of quality of food specially fishes. Microbiological as well as nutritional quality is very important factors for export and country consumption of fishes. In Bangladesh few studies have been done on microbiological in combination with nutritional aspects during preservation in low temperature of fish. At low temperature microbial and biochemical activities decreases.

1.3 Research Gap

Food security is a complex issue, where fish and fishery products are generally regarded as high risk commodity in respect of pathogen contents, natural toxins and other possible contaminants. Biological contaminants such as bacteria, viruses, protozoa, fungi and helminthes constitutes the major food borne dieases such as cholera, E. coli gastroenteritis, salmonellosis, shigellosis, campylobacteriosis, bucellosis, amoebiasis, typhoid fever and poilomyletis with varying degrees of severity, ranging from mild indisposition to chronic or life threading illness (Phyllis 2007). Some antimicrobial preservatives used to prolong the shelf-life of fishes are: acetic acid, lactic acid, polyphosphate, potassium sorbate, propionic

acid, citric acid, sodium acetate, sodium ascorbate, sodium chloride and sodium lactate. Antimicrobial properties vary with quality and quantity of preservatives, and time of exposure (Fernandez *et al.* 1998). Preservatives such as, sodium benzoate, citric acid, sorbatesand others are commonly used to improve the shelf life of salted, dried and smoked fisheries products (Espejo-Hermes 1998). The present study was carried out to assess the proximate composition and bacterial composition of fresh and salted hilsa (*Tenualosa ilisha*)

1.4 Research Needs

Bangladesh is in a vulnerable condition with global climate change and severe degradation of aquatic resources by human exploitation. In Bangladesh like many other countries in the world post harvest handling, processing and preservation of fish play a vital role in order to keep the fish in acceptable condition to the consumer. Currently keeping the quality of fish is one of the major problems in post techniques in fisheries (Tomiyasu and Zenitani 1957). From time immemorial humans discovered the way of long term preservation of fish by salting, drying and smoking. But preference for fresh and quality fish is increasing with the advancement of civilization. Therefore, proper information on the nutritional and microbial quality of *Tenualosa ilisha* in fresh and salted condition.

1.5 Objectives

The overall objective of the study is to investigate the microbiological and nutritional status of fresh and salted hilsa (*Tenualosa ilisha*)

The specific objectives are to –

1. Isolation and identification of pathogenic bacteria from frozen and salted Hilsa
2. Enumerate and compare the load of different heterotrophic and enteric bacteria
3. Determine the nutrient composition of *Tenualosa ilisha*

2. MATERIALS AND METHODS

2.1 Location of sampling:

Dhaka is the capital of Bangladesh and the principal city of Dhaka Division. Dhaka is a megacity and one of the major cities of South Asia. It is located on the banks of the Buriganga River. Dhaka, along with its metropolitan area, has an estimated population of over 12 million in 2008, making it the largest city in Bangladesh. It is the 9th largest city in the world and also 28th among the most densely populated cities in the world. (http://en.wikipedia.org).

2.2 Experimental Specimen

Hilsa, a tropical fish and the most popular and tasty fish of Bangladesh was considered as experimental fish. Identification of sample fish was done according to Rahman (2005).

2.2.1 Taxonomic Study of Hilsa

Phylum: Chordata
Class: Actinopterygii
Order: Clupeiformes
Family: Clupeidae
Genus: *Tenualosa*
Species: *Tenualosa ilisha* (Ham-Buch,1822)

(A) Fresh Hilsa Fish

(B) Salted whole Hilsa fish **(C) Salted slice Hilsa**

Fig.1 (A-C) Fresh and Salted samples of Hilsa (*Tenualosa ilisha*)

2.3 Collection of samples:

Samples were collected aseptically as far as practicable. During collection of sample, plastic bottles, markers, pen, field notebook, polythene bags were taken to the sampling sites for the purpose of sampling. Fish samples were collected in plastic jars. After collection, the samples were labelled properly and brought into the laboratory as soon as possible.

2.4 Measurement of pH and temperature of the samples:

pH of the samples was measured by a pH meter (Jenway 3310, U.K.) immediately after the collection while temperature was recorded by a mercury thermometer.

Table 2.1 location and sample types

Date	Location	Sample Type
21.08.13	Palashi Bazar	Salted Hilsa
09.09.13	karwan Bazar	Salted Hilsa
05.12.13	Ananda Bazar	Salted Hilsa
17.12.13	Anando Bazar	Fresh Hilsa
23.12.13	Palashi Bazar	Fresh Hilsa
2.01.14	Karwan Bazar	Fresh Hilsa
2.03.14	New Market	Fresh Hilsa
2.03.14	New Market	Salted Hilsa

2.5 Preservation of the sample:

Collected samples were preserved in a refrigerator at 4°C before and after the microbiological and nutritional analysis.

2.6 Media and techniques for the enumeration and isolation of bacteria

2.6.1 Culture and media:

Nutrient agar (NA) (Eklund and Lankford 1967) medium was used for the enumeration and isolation of aerobic heterotrophic bacteria present in fish samples while EMB agar (Scharlau Chemie S.A, Barcelona, Spain), SS agar (Techno Pharmachem, Bahadurgarh, Haryana), Manitol salt agar and TCBS agar (Scharlau Chemie S.A, Barcelona, Spain), were used to enumerate and isolate coliform bacteria, *Salmonella-Shigella, staphylococci* and enteropathogenic vibrios respectively. The pH of the medium was adjusted to 7.2 since most of the samples were within the range of 7.1-7.2 .The pH was adjusted before the addition of agar and sterilization.

2.6.2 Techniques employed:

Serial dilution technique (Greenberg *et al.* 1980) was used for the isolation of microorganisms. In fish sample, ten gm was diluted with 100 ml sterile distilled water in a sterile conical flask and shaken well. .This suspension was transferred to 9 ml of sterile water for ten-fold (1:10) dilution and further diluted up to 10^4 for the plating of NA medium and 10^3 dilutions for the plating of EMB agar medium and SS agar medium, Manitol salt agar medium, TCBS agar medium.

One ml of each of the diluted sample was taken in a sterilized Petri plate by sterilized pipette. Then molten agar medium poured and mixed thoroughly by rotating the Petri plate, first in one direction and then in the opposite direction. Plating in duplicated plates were made for each diluted sample. After setting the medium the plates were placed invertedly and incubated at 37°C for 24 h in an incubator (Memmert GmbH + Co Kg 8540 Sehwabach).

2.7 Enumeration of bacteria:

After 24 h of incubation the plates having well discrete colonies selected for counting from the respective culture plate. In case of EMB agar medium, pink or metallic green sheen colonies considered as coliform bacteria while white colonies considered as non lactose fermenter. The selected plates placed on colony counter (Digital colony counter, DC-8 OSK 100086, Kayagaki, Japan) and the colonies were counted.

2.8 Isolation of bacteria:

Based on their colonial morphology well discrete aerobic heterotrophic, coliform and related enteric bacterial colonies were selected immediately after counting. The selected colonies were isolated on slant for further studies.

2.9 Purification of the isolates:

After initial selection on the basis of growth pattern, the selected isolates purified through repeated plating (by streaking plate methods). When a plate yielded only one type of colonies the organisms considered to be pure.

2.10 Maintenance and preservation of isolates

The purified isolates then transferred on Nutrient agar, Eosin Methylene Blue agar, SS agar Manitol Salt agar and TCBS agar slant. The slants kept in polythene bags and preserved as stock culture in a refrigerator at 4°C for further study. Periodical transfers of isolates on agar slants were done for maintaining viability of the organisms.

2.11 Morphological observation of isolates

For the identification of selected isolates, following morphological characters were studied and recorded.

2.11.1 Colonial morphology:

The bacterial colonies on plating medium were morphologically studied as their form, elevation, margin, surface, pigmentation, opacity, whether grown inside, at the bottom or on the surface of the medium and their rate of growth (Eklund and Lankford 1967 Bryan 1950).

2.11.2 Microscopic examination of isolates:

Bacterial cells suspension made by using fresh culture with physiological saline. The prepared suspension used to make smear. A good quality glass slide was used for this purpose. Thin smear prepared on the clean and oil free slide. The smear allowed to dry in air and fixed by passing the slide over the flame of a spirit lamp. Two different staining methods *viz.* (i) Simple staining and (ii) Differential staining method employed to stain the fixed smears.

2.11.2.1 Simple staining:

Manual of Microbiological Methods (SAB 1957) was followed for simple staining. Basic dyes *viz.* crystal violet, basic fuchsin, safranin, mercurochrome and malachite green were used. The fixed smear flooded with a dye solution for one minute. The flooded smear washed off with water and dried in air.

2.11.2.2 Differential staining:

Staining procedures that make visible differences between microbial cells or parts of cells termed as differential staining (Pelczar *et al.* 1993). In differential staining process, a combination of dyes was used that take advantage of chemical differences among cells (Claus 1995). The differential stains most frequently used are the Gram stain, acid-fast stain and spore stain (Tortora *et al.* 1998). For this purpose, fixed smear exposed to more than one dye solution to differentiate cell and or its parts. In this study, two differential techniques were used *viz.* (i) Gram staining and (ii) Spore staining.

2.11.2.2.1 Gram staining:

This is one of the most important and widely used differential staining techniques which are considered as one of the important steps in identifying an unknown bacterium. For Gram staining, method described by (Claus 1995) was followed.

Fixed smear treated with the following solutions and after application of each solution; the slide gently washed off with water.

Crystal violet 60 sec., Lugol's iodine solution 60 sec., 95% Ethyl alcohol less than 30 sec., Mercurochrome solution 60 sec. The slide was blotted dry and observed under microscope (Nikon Microphot, UFX-IIA, Japan).

The results were recorded as Gram positive (blue-violet) and Gram negative (light red).

2.11.2.2.2 Spore staining:

The method described by (Claus 1995) was applied in spore staining. Smear made from 16-18 h old bacterial culture. The fixed smear flooded with 5% aqueous solution of malachite green and heated over a brass plate for about 15 minutes taking care that the dye must not be dried off. Excess dye then washed gently and safranin used as a counter stain for 1 minute. The slide washed gently, dried and examined under microscope.

Spores were stained with green color of malachite green and vegetative cells or sporangia were stained with red color of safranin. The shape and position of the spores within sporangia observed. The swelling nature of the sporangium also observed and recorded.

2.11.2.3 Negative staining (SAB 1957):

To introduce a rapid method for demonstrating microorganisms against a dark background and for use in measuring the size of bacteria an unusual staining process are employed. This process is known as Banians' Congo red method.

In this method a drop of 2% Congo red [2 g Congo red (80% dye content) and 100ml distilled water] placed on a slide and the culture were mixed with a loop and spread out in a thick film. After drying, the film was washed with 1% HCl. The red coloured smear was then changed into blue. The slide with blue film was examined under microscope.

Unstained cells with blue background observed under microscope.

2.11.2.4 Wet mount method (Claus 1995):

Bacterial suspensions were prepared using 18 h cultures with physiological saline (0.85%NaCl). On a clean and oil free slide a drop of bacterial suspension was taken and covered with a clean cover slip and observed under phase contrast microscope. This was used for the study of vegetative cells, spores, sporangia and motility. Photomicrographs were taken with a advanced research microscope (Nikon Microphot, Japan) fitted with photo micrographic attachment (Nikon, FX 35 WA, Japan).

2.11.3 Measurement of bacterial cells:

Vegetative cells of bacterial isolates were measured. The measurement was done by comparing the photographs of bacterial cells with that of the stage micrometer. The compared photographs were of same magnification. (One division of the stage micrometer is equal to ten μm).

2.12 Physiological and biochemical studies of the isolates:

Following Bergey's Manual vol. 1 (Kreig and Holt 1984) and vol.2 (Sneath *et al.* 1986), Manual of Microbiological Methods (SAB 1957), Microbiological Methods (Collins and Lyne 1984) and Understanding Microbes (Claus 1995) the following important physiological and biochemical tests of the isolated bacteria were carried out.

2.12.1 Fecal coliform test (APHA 1998):

To make a distinction between the coliform to fecal and non fecal origin on the basis of elevated temperature this test performed. For this purpose lactose broth with Durham's tube was inoculated with the test isolates. After 24 hours of incubation at 44.5^{o}C in water bath the positive fecal coliform showed gas formation in Durham's tube.

2.12.2 Catalase test (Claus 1995):

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS).

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \uparrow$$

To demonstrate catalase activity, test organisms were taken by a sterilized loop on a glass slide and a drop of hydrogen peroxide was added to each of them.

The evolution of bubbles indicated the positive result *i.e.* the organism having the enzyme catalase.

2.12.3 Deep glucose agar test (SAB 1957):

Microorganisms vary widely in their requirements for oxygen. The nature of microbial growth in agar deeps reflects the cells' relative need for oxygen or an oxygen free

environment. In relation to free oxygen, organisms are generally classified as strict aerobes, microaerophiles, facultative anaerobes and strict anaerobes.

A tube of deep glucose agar medium (Hall 1929) was inoculated in fluid condition approximately at 45°C. The tube rotated to mix the inoculums with the medium and allowed to solidify.

Observation was made after incubation at 37°C for 7 days to find out whether the organisms grew on the surface and in the upper layer of the medium (strict aerobes), or the organisms grew just a few millimeters below the surface (microaerophiles), or the organisms grew throughout the medium (facultative anaerobes), or the organisms grew deeper in the medium (strict anaerobes).

2.12.4 Voges-Proskauer (V. P.) Test (Sneath *et al.* 1986)

Voges-Proskauer (V.P.) test is a color reaction test for the production of a neutral product during glucose fermentation by microorganisms. Acetoin or acetyl-methyl carbinol oxidised to diacetyl, which reacts with creatine and forms a red complex.

For this test VP broth tubes inoculated and incubated for 5 days at 37°C. When sufficient growth observed, 3 ml of 5% alcoholic α-napthol solution added to each tube followed by 1 ml of 40% potassium hydroxide and 0.3% creatine solution. The tubes were then shaken vigorously and allowed to stand.

Development of crimson to ruby red color indicates a positive reaction that is the production of acetyl-methyl carbinol.

2.12.5 Methyl red test (Sneath *et al.* 1986):

Methyl red (M.R.) test is the test for mixed acid fermentation of glucose by microorganisms. Excreted acid contains large amount of formic, acetic, lactic and succinic acid and causes a major decrease in pH that can be detected by "Methyl Red" indicator. For this test V.P. broth was inoculated and incubated at 37°C for 5 days.

After incubation 5 drops of methyl red indicator added to the culture broth. Red color throughout the broth indicated positive reaction whereas yellow or any yellowish red indicated negative reaction.

2.12.6 Hydrolysis of casein (Sneath *et al.* 1986)

Casein is a milk protein and it comprises about 85% of the total protein in milk. Many microorganisms have the capacity to hydrolyze casein. This test demonstrates the ability of microbes to degrade casein into soluble peptides and amino acids by the enzyme caseiase.

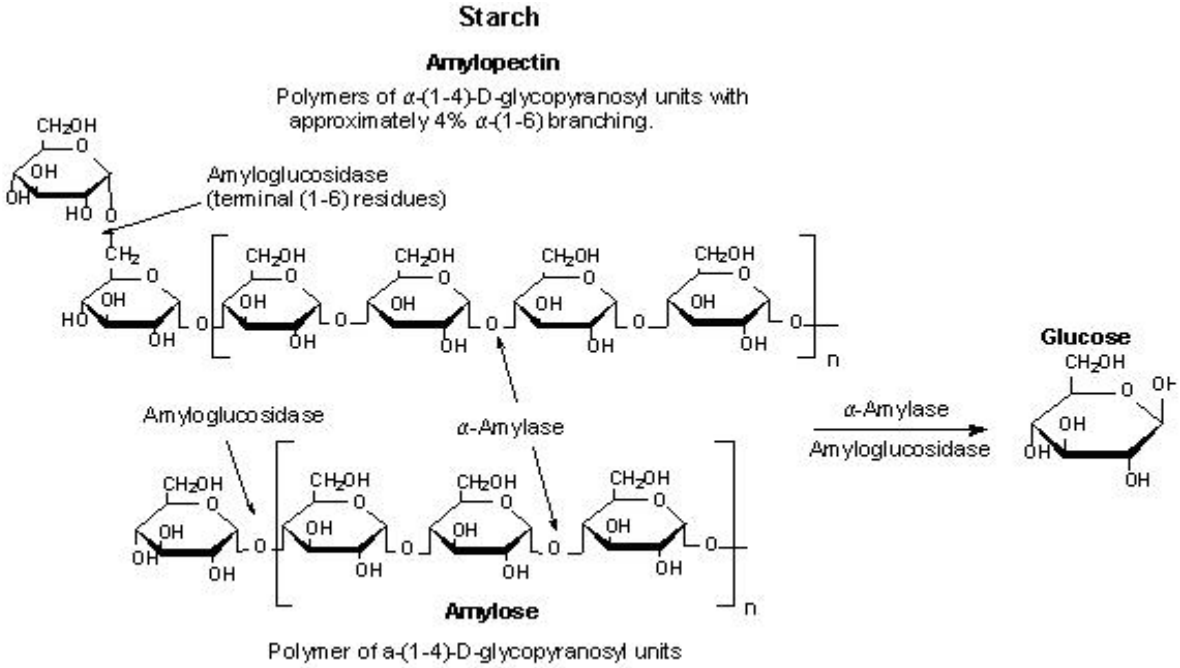
One ml of sterilized skim milk was taken in a sterilized Petri-plate and then melted agar medium poured and mixed thoroughly (Collins and Lyne 1984). After solidifying, the plates inoculated and incubated at 37°C for 48 h.

Formation of a clear, transparent zone around the growth indicated hydrolysis of casein.

2.12.7 Hydrolysis of starch (Claus 1995):

Organisms capable of hydrolyzing starch to form monosaccharide or disaccharide possess the enzyme amylase. As an extracellular enzyme, amylase diffuses outward from the bacterial cells and breakdown starch. This test revealed the presence or absence of the enzyme amylase in the organisms. For this test, starch-agar plates inoculated with test organisms and the plates incubated at 37°C for 48 h. After incubation, the surface of these plates flooded with iodine solution.

Iodine reacts with starch and form starch iodide and gives the colour deep blue. Development of a clear zone around the growth indicated starch hydrolysis.



2.12.8 Utilization of Citrate (Atlas 1997):

This test demonstrates the ability or inability of test organisms to use citrate as sole source of carbon for metabolism and growth. Tubes containing Simmon's citrate agar inoculated and incubated at 37°C for 4 days.

Utilization of citrate established by changing the color from green to blue.

2.12.9 Utilization of propionate (Sneath *et al.* 1986):

Propionate agar slants were inoculated with 24 h old culture and incubated at 37°C for 3-5 days.

Production of a pink color indicates the utilization of propionate by bacteria.

2.12.10 Nitrate reduction test (SAB 1957):

Nitrate reduction is evident by complete or partial disappearance of nitrate accompanied by appearance of nitrite, ammonia or free nitrogen. This test performed to observe the organisms' capability on the reduction of nitrate to nitrite. The formation of nitrite indicated the presence of the enzyme nitrate reductase in the organisms.

The following three reagents were required for this test:

Reagent A: Sulfanilic acid – acetic acid solution:

Sulfanilic acid - 8.0 g

5N acetic acid - 1000 ml

(1 part chemically pure acetic acid 2.5 parts distilled water).

Sulfanilic acid was dissolved in acetic acid and stored in brown glass bottle.

Reagent B: Dimethyl-α-napthalamine solution:

Dimethyl-α-napthalamine - 6.0 ml

5N acetic acid - 1000 ml

Stored in brown glass bottle.

Reagent C: Zinc dusts

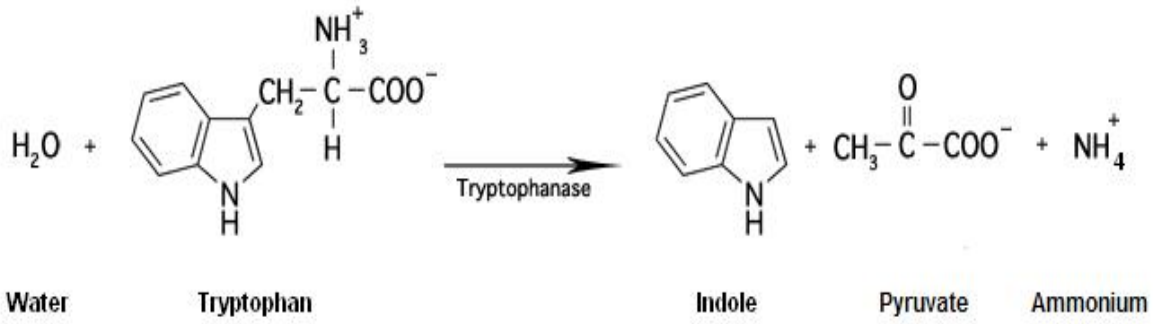
The tubes of nitrate broth in duplicates inoculated with test organisms and then incubated at 37°C for 72 h. After incubation, 1 ml of **reagent A** was added to the incubated tube and shaken. Then 1 ml of **reagent B** was also added to each tube and shaken well.

Formation of a distinct red or pink color indicated the reduction of nitrate to nitrite. Absence of nitrite may be due to complete conversion of nitrate as well as no reduction at all. A pinch of zinc dust then added to the tube showing absence of nitrite and it was allowed to stand for a few minutes. Any remaining nitrate (in case) would be reduced to nitrite by zinc and the characteristic pink or red color would appear and no color indicated complete reduction.

2.12.11 Production of indole (Atlas 1997):

Indole is generated by reductive deamination from tryptophan via the intermediate molecule indolepyruvic acid. Tryptophanase catalyzes the deamination reaction, during

which the amine (-NH_2) group of the tryptophan molecule is removed. Final products of the reaction are indole, pyruvic acid, ammonia (NH_3) and energy.



For this test, Kovac's modification of Ehrlichs and Bohme method (1905) was followed. In this method 1% tryptone broth medium was used. The inoculated tubes incubated at 37° C for 3 days. After incubation 2 ml of the test reagent (Kovac's reagent) was added.

A rose pink color indicated formation of indole.

2.12.12 Oxidase test (Claus 1995):

The enzyme oxidase, present in certain bacteria catalyses the transport of electron from donor bacteria to the redox dye tetra-methyl-para-phenylene-diamine dihydrochloride. The dye in the reduced state has a deep purple color. To perform this test filter papers soaked in 1% aqueous tetramethyl-phenylene-diamine dihydrochloride. Fresh young culture rubbed on the filter paper with a clean glass rod. Results were recorded within 10 seconds. **Blue color indicated a positive result.**

2.12.13 Kligler's Iron Agar (KIA) test (Atlas 1997):

Kligler's Iron Agar medium used to differentiate Gram-negative enteric bacteria or their ability to ferment dextrose or lactose and their production of hydrogen sulfide.

Tubes of KIA media were inoculated by stabbing the butt and streaking the slant with inocula of 24 h. The inoculated media then incubated at 37 °C for 48 h.

Yellow color in the butt and slant indicated acid production while hydrogen sulfide production indicated by blackening of slant. Break in the medium indicated gas formation. Red color in the butt and slant indicate alkaline reaction.

2.12.14 Motility test by wet mount method:

Wet mount slide was prepared by placing bacterial suspension on a clean slide and covered by cover glass. The edge of the cover glass was sealed with petroleum jelly/nail polish and the bacterial cells examined with a phase contrast microscope.

Movement of the bacterial cells revealed the motility of bacteria and the result recorded.

2.12.15 Urease production test (Rustigen and Stuart 1941):

A modified YS broth with 0.5% yeast extract and 0.0012% phenol red (w/v) was prepared in flasks and flasks were sterilized. Urea was filter sterilized and then added to the above medium making 2% concentration of urea and finally the medium dispensed into sterile test tubes. The test cultures inoculated and incubated at 37°C. Control tubes containing the basal medium (without urea) inoculated and with urea not incubated.

An increase in alkalinity indicated by magenta red color was regarded the presence of urease.

2.12.16 Potassium hydroxide solubility test (Schaad 1988):

The test was done with a 3% potassium hydroxide (KOH) solution (Suslow *et al.* 1982). One to two drops of 3% KOH placed on a clean and dried glass slide. A loop full of the bacterial cells from the edge of a 24 h old culture transferred and mixed thoroughly with the drops of KOH on the slide for 10 sec.

The organism considered positive when KOH solution become viscous and showed a slimy thread and it was negative when there was no slime. The organisms showing positive reaction to KOH were Gram-negative bacteria and vice-versa.

2.12.17 Growth response of the isolates at different concentrations of NaCl (Sneath *et al.* 1986):

To observe the growth response at different salt concentration of the isolates nutrient broth with different concentrations of NaCl *viz*. 2, 5, 7 and 10% were used. Test tubes inoculated with 24 h bacterial culture and incubated at 37°C for 48 h. Turbidity of inoculated broth indicated the growth of the isolates.

Using spectrophotometer (Shimadzu, UV-120-02, Japan) turbidity measured by the percentage of light absorption at 600 nm (Oscariz and Pisabarro 2000).

2.13 Identification of the isolates:

Gram positive bacteria were provisionally identified following Bergey's Manual of Systematic Bacteriology (Sneath *et al.* 1986). Enteric and related bacteria were identified by using Manual for Laboratory Investigations of Acute Enteric Infections (WHO 1987) and Bergey's Manual of Systematic Bacteriology (Krieg and Holt 1984).

2.14 Determination of Proximate Composition of Fish Sample

Fresh and raw fishes were analyzed for their nutrient content in order to have a clear idea about the category of fish where it belongs to. For example hilsa fish which is very popular fish is a fatty species of fish and for that reason it is not fit for drying process. It can only be processed and preserved by freezing and by salting. To see the relationship among the nutrient contents of the sampled fish and shrimp, the following procedure had been followed.

2.14.1 Estimation of moisture content

Moisture content is expressed as the amount of water as percentage (%). Moisture content was determined by oven drying method (AOAC 1980). Pre weighted samples were oven dried (95-105^{0}C) using pre weight foil paper boat.

The percentage of moisture content was calculated by following equation:

Weight of the dish = W_0

Weight of the dish +Wet sample = W_1

Weight of the dish +Dry sample = W_2

$$\text{Moisture content of the sample (\%)} = \frac{\{(W1 - W0) - (W2 - W0)\}}{(W1 - W0)} \times 100$$

Dry matter content of the sample (%)= 100- moisture

Moisture factor = (100 – moisture) / 100

2.14.2 Estimation of crude protein

Crude protein content of fish was determined by micro-kjeldal method using Kjeltec machine (Model Tecator Kjeltec System 1026 Manual 1987). The basic principal of this method involves the conversion of nitrogen of protein into NH_4SO_4 when boiled with H_2SO_4, which on distillation with excess of sodium hydroxide gives ammonia, which is absorbed in boric acid solution containing methyl red. The amount of nitrogen absorbed in boric acid is determined by tritation with 0.01N HCl. Protein conversion factor (6.25) was used in converting nitrogen to crude protein. The percentage of nitrogen in the sample was calculated by using the following formula:

$$\text{\% of nitrogen} = \frac{(S - B) \times A \times C \times 100}{\text{Weight of sample} \times 1000}$$

S = Titration reading for sample

A = Strength of 0.01N HCl (0.01)

B = Titration reading for blank

C = Digest taken for distillation (dilution factor) ≈ 20

% crude protein (fresh sample) = N_2 x 6.25 x moisture factor

2.14.3 Estimation of fat content

Lipid content was determined by extraction with a mixture of chloroform (2 : 1) to a little amount of sodium chloride (0.9%) as recommended by AOAC (1975). About 1g of dried sample was taken in a conical flask and chloroform methanol mixture was added .The mixture was allowed to stand overnight and lower lipid protein was transferred to a pretreated and weighed flask filtered through a filter paper and heated to dryness. The difference in the two weights of the round joint flask gave the weight of the fat (Folch 1957) .Finally the fat was calculated in following way:

$$\text{\% of fat} = \left(\frac{W2 - W1}{S} \times 100\right) \text{ x moisture factor}$$

W_2 = Final weight of the conical flask

W_1 = Initial weight of the empty conical flask

S = Weight of the sample taken

2.14.4 Determination of ash

Ash content was determined by ignition of samples in a muffle furnace at 550 - 600^{0}C for 16 hours (AOAC 1980).

The percentage of ash is calculated as follows:

Weight of the clean dry crucible = W_0

Weight of the clean dry crucible + dry sample = W_1

Weight of the clean dry crucible + ash = W_2

Ash content of the fresh sample (%) = $\left(\frac{W2 - W0}{W1 - W0} \times 100\right) \times$ moisture factor

2.15 Statistical Analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) v. 16.0 for windows (SPSS, SAS Institute Inc. Cary, USA). The data were analyzed to determine the descriptive statistics such as Standard Error of Mean (SEM), Standard Deviation (SD), Statistic Mean, Minimum and Maximum value and Ranges of variables. One way ANOVA and Duncan multiple test were done to test the significance between two floodplains using 5% level of significance.

3. RESULTS

In the present study, fresh hilsa and salted hilsa were examined microbiologically and proximate compositions to make a comparative study between fresh and salted conditions. Samples were collected from four different market of Dhaka city. The present study has been delineated in three distinct phases.

- ✓ Quantitative analysis of microorganisms associated with the collected fresh and salted hilsa fish samples.
- ✓ Isolation and identification of the heterotrophic and enteropathogenic bacteria associated with the collected samples.
- ✓ Analysis of biochemical conditions such as crude protein, moisture, lipid and ash present in the fresh and salted hilsa fish samples.

The collected samples were analyzed in various aspects such as microscopic observation, cultural and biochemical tests to isolate and identify the organisms of interest.

3.1 Temperature and pH of the collected samples

The temperature, pH and bacterial load of the fish samples were shown in Table 3.1. During the present investigation temperature was observed of the collected fish samples. The pH of the sample of fresh hilsa fish ranged between 6.52 to 6.70 and salted hilsa fish 5.13 to 5.70.

Table.3.1 Temperature and pH of the collected samples.

Sampling sites	Sampling types	Temperature	pH
Palashi Bazar	Salted Hilsa	28.3	5.13
Karwan Bazar	Salted Hilsa	28.6	5.70
Ananda Bazar	Salted Hilsa	28.0	5.52
Anando Bazar	Fresh Hilsa	29.1	6.52
Palashi Bazar	Fresh Hilsa	30.2	6.70
Karwan Bazar	Fresh Hilsa	30.2	6.76
New Market	Fresh Hilsa	29.8	6.70
New Market	Salted Hilsa	31.2	5.62

3.2 Bacterial load of the collected samples

The heterotrophic bacterial load of fresh fish samples on nutrient agar was ranged between 1.4×10^4 cfu/g to infinity. For the isolation of *Salmonella* and *Shigella*, the selective medium SS agar was used and the count was ranged between 2×10^2 to 6.35×10^3 cfu/g. Enteric bacteria with special reference to coliform count on EMB agar ranged between 8.5×10^4 to 1.2×10^6 cfu/g, Manitol salt agar was used to assess and isolate *Staphylococcus* and the count ranged between 2.5×10^2 to 1.76×10^5 cfu/g and only one colony found in TCBS agar plate. .

The bacterial load of salted fish samples on nutrient agar ranged between 1.4×10^4 cfu/g to 7.45×10^4 cfu/g. Enteric and related bacterial count on EMB agar ranged between 5.2×10^3 to 8.5×10^3 cfu/g. Bacterial count on Manitol salt agar was ranged between 1×10^2 to 4×10^4 cfu/g and any bacterial colony found in TCBS agar and SS agar plate.

Table3.2 Total bacterial count (mean ± SEM) in *Tenualosa ilisha* fish sample

Source	Type of fish	
	Fresh	Salted
Palashi Bazar	$9.8 \pm 1.2 \times 10^{5aA}$	$1.4 \pm 0.6 \times 10^{4b}$
Karwan Bazar	$1.22 \pm 0.12 \times 10^{6aA}$	$7.45 \pm 0.55 \times 10^{4b}$
Anando Bazar	$9.3 \pm 1.7 \times 10^{5aA}$	$6.85 \pm 3.15 \times 10^{4b}$
New Market	$6.6 \pm 0.2 \times 10^{4B}$	$7.0 \pm 1.0 \times 10^4$

Means followed by different superscript small letters indicate significant difference within rows and capital letters denote significant difference within columns (ANOVA, HSD; $P<0.05$)

The table 3.2 describes the differences in mean according to row and column. The height count of TBC was $1.22 \pm 0.12\times10^6$ in fresh sample in Karwan Bazar and lowest TBC count was $1.4 \pm 0.6\times10$ in salted sample in Palashi Bazar.

Table 3.3 Total EMB count (mean ± SEM) in *Tenualosa ilisha* fish sample

Source	Type of fish	
	Fresh	Salted
Palashi Bazar	$2.15 \pm 0.15 \times 10^{5bc}$	$5.2 \pm 0.3 \times 10^{3c}$
Karwan Bazar	$1.2 \pm 0.1 \times 10^{6aA}$	$8.5 \pm 1.5 \times 10^{3cC}$
Anando Bazar	$2.66 \pm 0.49 \times 10^{5bB}$	$6.4 \pm 0.4 \times 10^{3cC}$
New Market	$1.9 \pm 0.4 \times 10^{5bc}$	$7.75 \pm 1.7 \times 10^{4bc}$

Means followed by different superscript small letters indicate significant difference within rows and capital letters denote significant difference within columns (ANOVA, HSD; P<0.05)

In this study, the highest EMB count was $1.2 \pm 0.1 \times 10^6$ in fresh hillsa collected from Karwan Bazar and lowest was $5.2 \pm 0.3 \times 10^3$ in salted sample collected from New market

Table 3.4 Total MSA count (mean ± SEM) in *Tenualosa ilisha* fish sample

Source	Type of fish	
	Fresh	Salted
Palashi Bazar	$5.1 \pm 1.1 \times 10^{4B}$	$2.1 \pm 0.2 \times 10^{3}$
Karwan Bazar	$1.26 \pm 0.06 \times 10^{5aA}$	$4.0 \pm 1.0 \times 10^{4b}$
Anando Bazar	$1.64 \pm 0.24 \times 10^{5aA}$	$3.2 \pm 0.8 \times 10^{3b}$
New Market	$2.5 \pm 0.5 \times 10^{2B}$	$1.5 \pm 0.5 \times 10^{2}$

Means followed by different superscript small letters indicate significant difference within rows and capital letters denote significant difference within columns (ANOVA, HSD; P<0.05)

The table 3.4. shows TSC (total Staphylococcul count) in fresh and salted hilsa in different market.Among the four places, height TSC value was found$1.64 \pm 0.24 \times 10^5$ in fresh sample collected from Anando Bazar and lowest TSC was found $1.5 \pm 0.5 \times 10^2$ in Salted sample collected from New Market.

Table 3.5 Total *Shalmonella-Shigella* count (mean ± SEM) in *Tenualosa ilisha* fish sample

Source	Type of fish	
	Fresh	Salted
Palashi Bazar	$4.9 \pm 1.1 \times 10^{3A}$	0
Karwan Bazar	$6.35 \pm 1.35 \times 10^{4A}$	0
Anando Bazar	$1.5 \pm 0.5 \times 10^{2B}$	0
New Market	0	0

Means followed by different superscript small letters indicate significant difference within rows and capital letters denote significant difference within columns (ANOVA, HSD; $P<0.05$)

In table 3.5 shows that no *Shalmonella-Shigella* was observed in salted fish sample. Among the fresh samples highest value found 6.35 ± 1.35×10 in Karwan bazaar.

Table 3.6 Total TCBS count (mean ± SEM) in *Tenualosa ilisha* fish sample

Source	Type of fish	
	Fresh	Salted
Palashi Bazar	$5.0 \pm 5.0 \times 10^{2}$	0
Karwan Bazar	$5.0 \pm 5.0 \times 10^{2}$	0
Anando Bazar	0	0
New Market	0	0

In the present study, *Vibrio* is present in only two samples of fresh hilsa collected from Kawran Bazar and Ananda Bazar.

3.3 Isolation and purification of the selected isolates.

During this study a total of 66 colonies were primarily selected from different isolating medium. Among 66 colonies 26 isolates were selected and finally 24 isolates were selected and purified for detail study towards identification. Out of these 24 isolates 12 were Gram positive bacteria and remaining 12 were Gram negative bacteria. Bacterial colonies developed on the pour plate and streak plate techniques were shown in Plate. 3.1 & 3.2.

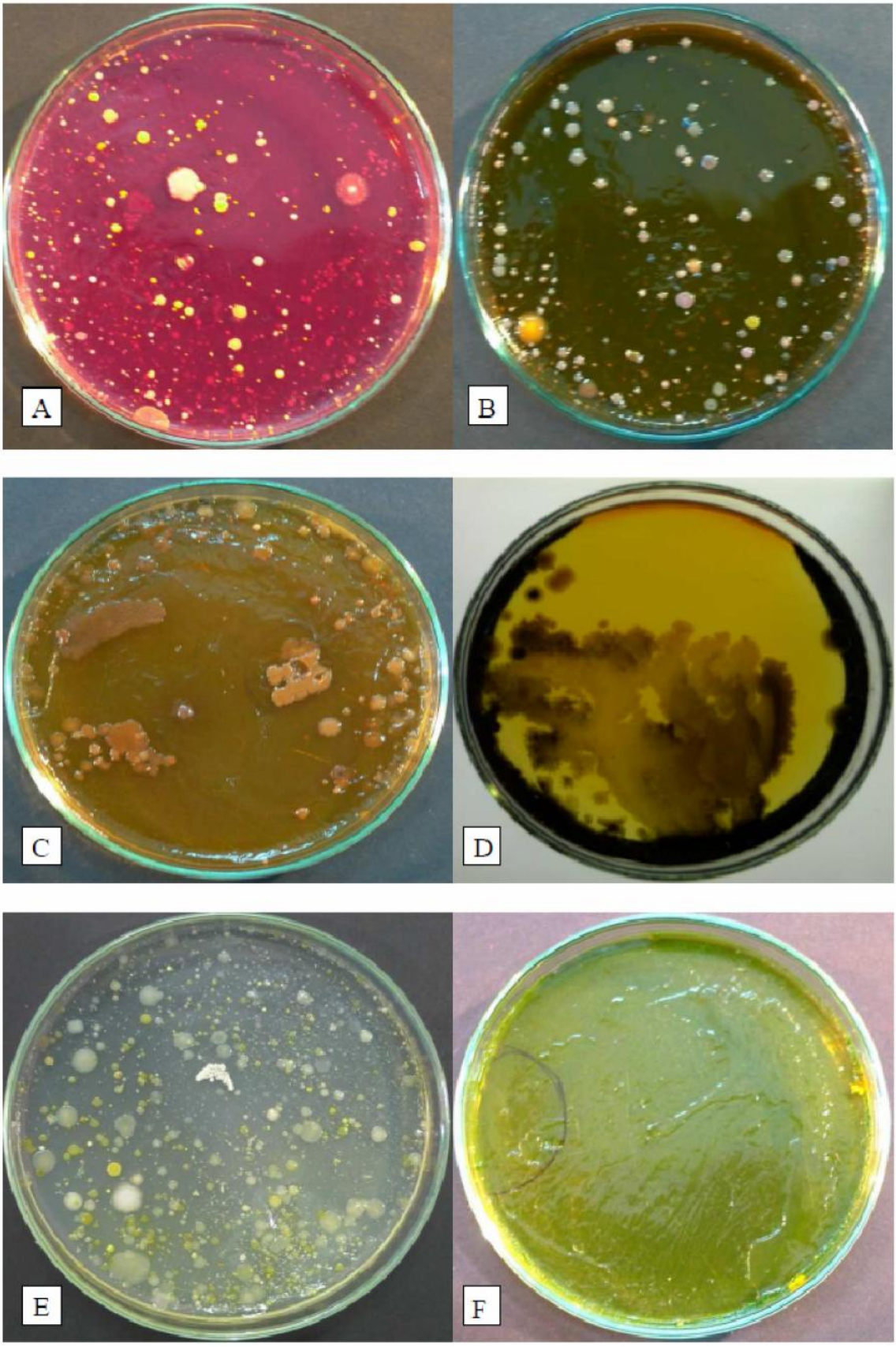


Plate. 3.1. Photographs showing bacterial colonies of different steps of enumeration and isolation. A. Manitol salt agar plate, B. EMB agar, C & D. SS agar, E. Nutrient agar and F. TCBS agar plate.

3.4 Colonial morphology of the selected isolates.

Colonies of the selected isolates were found to be different in their form, elevation, margin, surface, colour and optical characteristics. The colonial morphology of the selected Gram positive isolates were presented in Table 3.2 and the colonial morphology of the Gram negative isolates were presented in Table 3.3. Most of the colonies of the isolated bacteria were entire, flat, smooth and off-white in nature.

3.5 Microscopic observation of the selected isolates.

Among the Gram positive bacterial isolates 3 were rod shaped, spore former and reaming 8 isolates were round shaped. On the other hand the Gram negative bacteria were short rod and non- spore former. Microscopic observation of the selected isolates was shown in the Table 3.7 & 3.8. Photomicrographs of the selected isolates were shown in the Plate. 3.3 and 3.4

Table.3.7 Colonial morphology of the selected Gram positive isolates

Isolate number	Size	Shape	Pigmentation	Elevation	Margin	Surface	Optical character	Diameter in mm
S3/NAD2/1	Large	Round	Off white	Convex	Entire	Smooth	Opaque	5.2
S2/NAD2′/1	Small	Round	Yellow	Convex	Entire	Smooth	Opaque	2.5
S4/NAD2/1	Small	Round	Yellow	Convex	Entire	Smooth	Opaque	1.5
S3/NAD3’/1	Small	Round	Off white	Flat	Entire	Smooth	Opaque	2.3
S6/NAD1/1	Small	Round	Yellow	Convex	Entire	Smooth	Opaque	0.5
S3/MSAD1/1	Small	Round	White	Convex	Entire	Smooth	Opaque	1.1
S3/MSAD1′/1	Small	Round	White	Convex	Entire	Smooth	Opaque	1.3
S4/MSAD2/1	Small	Round	Pink	Convex	Entire	Smooth	Opaque	1.0
S4/MSAD2/2	Small	Round	Light Pink	Convex	Entire	Smooth	Opaque	0.6
S5/MSA1/2	Small	Irregular	Light Pink	Convex	Entire	Smooth	Opaque	0.3
S5/MSA/2/2	Small	Round	Yellow	Convex	Entire	Smooth	Opaque	1.5
S2/EMB/2/2	Small	Round	Off white	Raised	Entire	Smooth	Opaque	0.5

Table 3.8 Colonial morphology of the selected Gram negative isolates

Isolate number	Size	Shape	Pigmentation	Elevation	Margin	Surface	Optical character	Diameter in mm
S5/MSAD1/1	Medium	Irregular	Yellow	Convex	Entire	Smooth	Opaque	5.2
S3/EMBD1´/1	Small	Round	Pink	Flat	Entire	Smooth	Opaque	1.2
S4/EMBD1/6	Large	Irregular	Off white	Convex	Entire	Smooth	Opaque	8.0
S4/EMBD1/2	Medium	Irregular	Off white	Convex	Entire	Smooth	Opaque	4.2
S6/EMBD1'/1	Large	Round	Light pink	Convex	Entire	Smooth	Opaque	2.7
S6/EMBD2/1	Medium	Round	Blackish	Convex	Entire	Smooth	Opaque	4.0
S3/EMBD2/2	Small	Round	Ember	Convex	Entire	Smooth	Opaque	0.3
S4/EMBD1/4	Small	Irregular	Brown	raised	Entire	Smooth	Opaque	3.5
S4/TCBS/1	Small	Round	Yellow	Convex	Entire	Smooth	Opaque	0.1
S6/TCBS/1	Small	Round	Yellow	Convex	Entire	Smooth	Opaque	0.3
S5/SSD1/2	Medium	Round	Ember	Convex	Entire	Smooth	Opaque	4.5
S4/SSD2/1	Medium	Round	Black	Convex	Entire	Smooth	Opaque	2

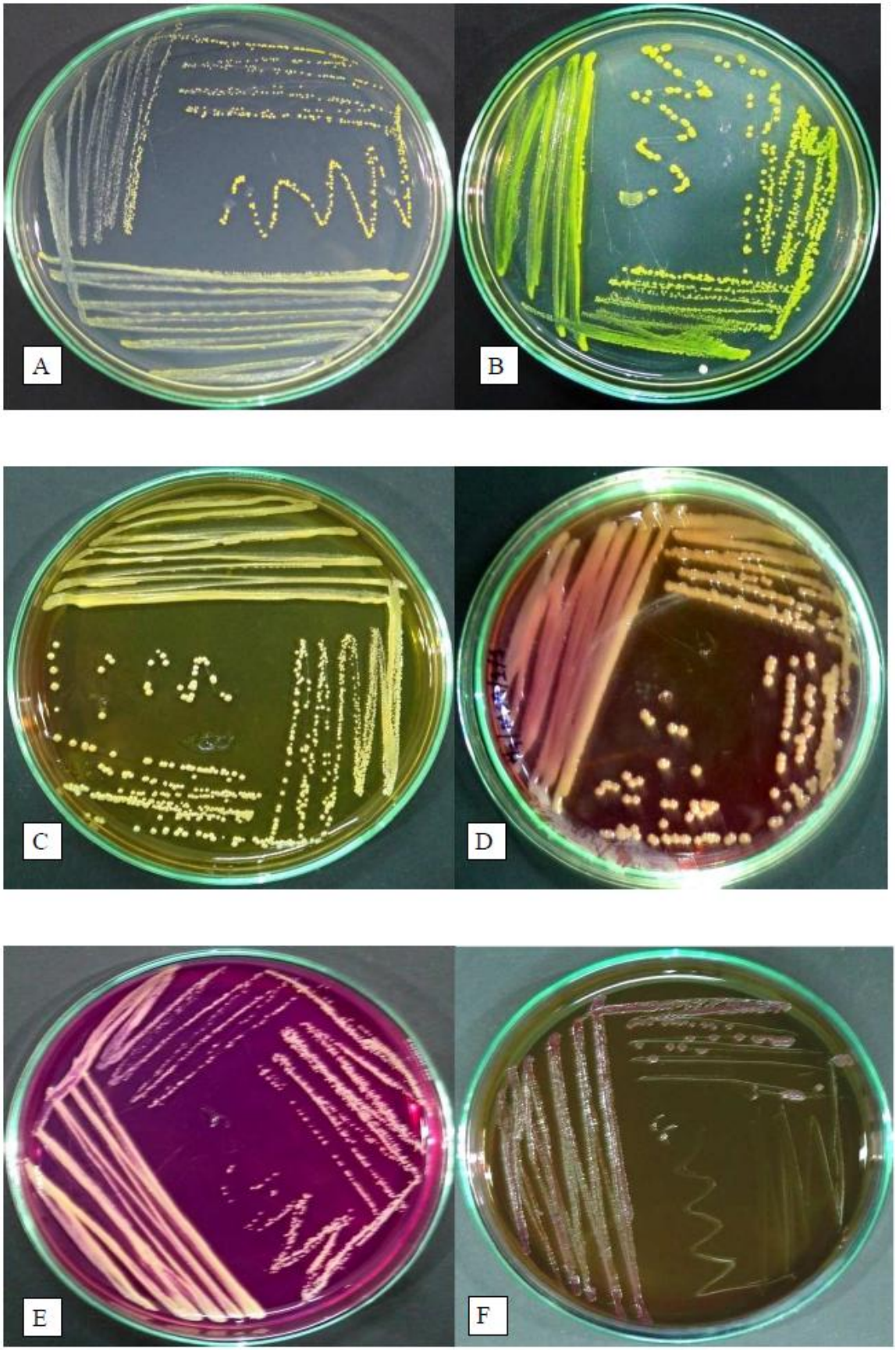


Plate.3.2 Purification of selected isolates by streak plate technique . A & B. Nutrient agar plate, C, D & E. MSA plate, F. SS agar plate

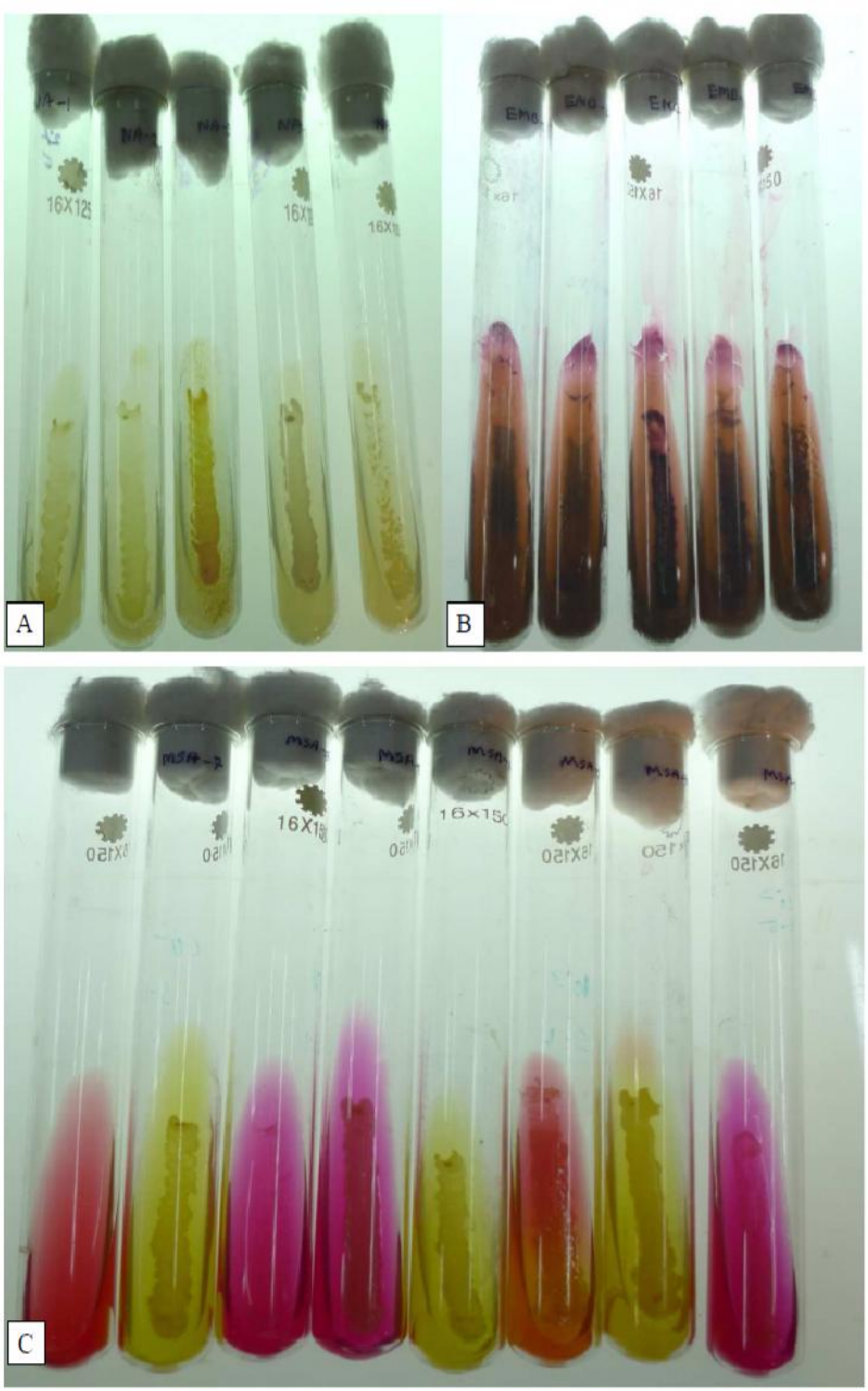


Plate 3.3 Growth of selected isolates on different culture media. A. Nutrient Agar, B. EMB Agar and C. MSA medium, bacteria change color pink to yellow.

Table 3.9 Microscopic studies of the isolated Gram positive bacteria.

Isolate No	Vegetative cells	Spore	Sporangia
S2/NA/2/1	Coccus, occur in cluster	Non-spore former	Absent
S1/NA/2′/1	Coccus, occur in clusters, tetrads.	Non-spore former	Absent
S3/NA/2/1	Coccus, occur in chains.	Non-spore former	Absent
S2/NA/3/1	Short rods, occur singly.	Ellipsoidal	Swollen
S5/NA/1/1	Short rods, rounded ends, occur singly	Non-spore former	Absent
S2/MSA/1	Coccus, occur singly	Non-spore former	Absent
S2/MSA/1′/1	Coccus, occur singly	Non-spore former	Absent
S3/MSA/2/1	Short rod, rounded end, occur singly.	Non-spore former	Absent
S3/MSA/1/1	Coccus, occur as diads.	Non-spore former	Absent
S4/MSA/1/2	Coccus, occur as diads.	Non-spore former	Absent
S5/MSA/2/1	Coccus, rounded end,occurs singly	Non-spore former	Absent
S2/EMB/2	Rod, rounded end, occur singly	Spore former	Swollen

Table 3.10 Microscopic studies of the isolated Gram negative bacteria.

Isolate No	Vegetative cell	Spore
S2/EMB/2'/1	Short rod, rounded end, occurs singly	Non spore former
S3/EMB/1/6	Short rod, rounded end, occurs singly.	Non spore former
S3/EMB/1/2	Short rod, rounded ends, occur singly	Non spore former
S5/EMB/1'/1	Short rod, rounded ends, occur singly	Non spore former
S5/EMB/2/1	Short rod, rounded ends, occur singly	Non spore former
S2/EMB/2/2	Long rod, rounded ends, occur singly	Non spore former
S3/EMB/1/4	Long rod, rounded ends, occur singly	Non spore former
S3/TCBS/1	Short rod, rounded ends, occur singly	Non spore former
S5/TCBS/1	Short rod, rounded ends, occur singly	Non spore former
S4/SS/1/2	Short rod, rounded ends, occur singly	Non spore former
S3/SS/2/1	Short rod, rounded ends, occur singly	Non spore former
S4/MSA/1/2	Short rod, rounded ends, occur singly	Non spore former

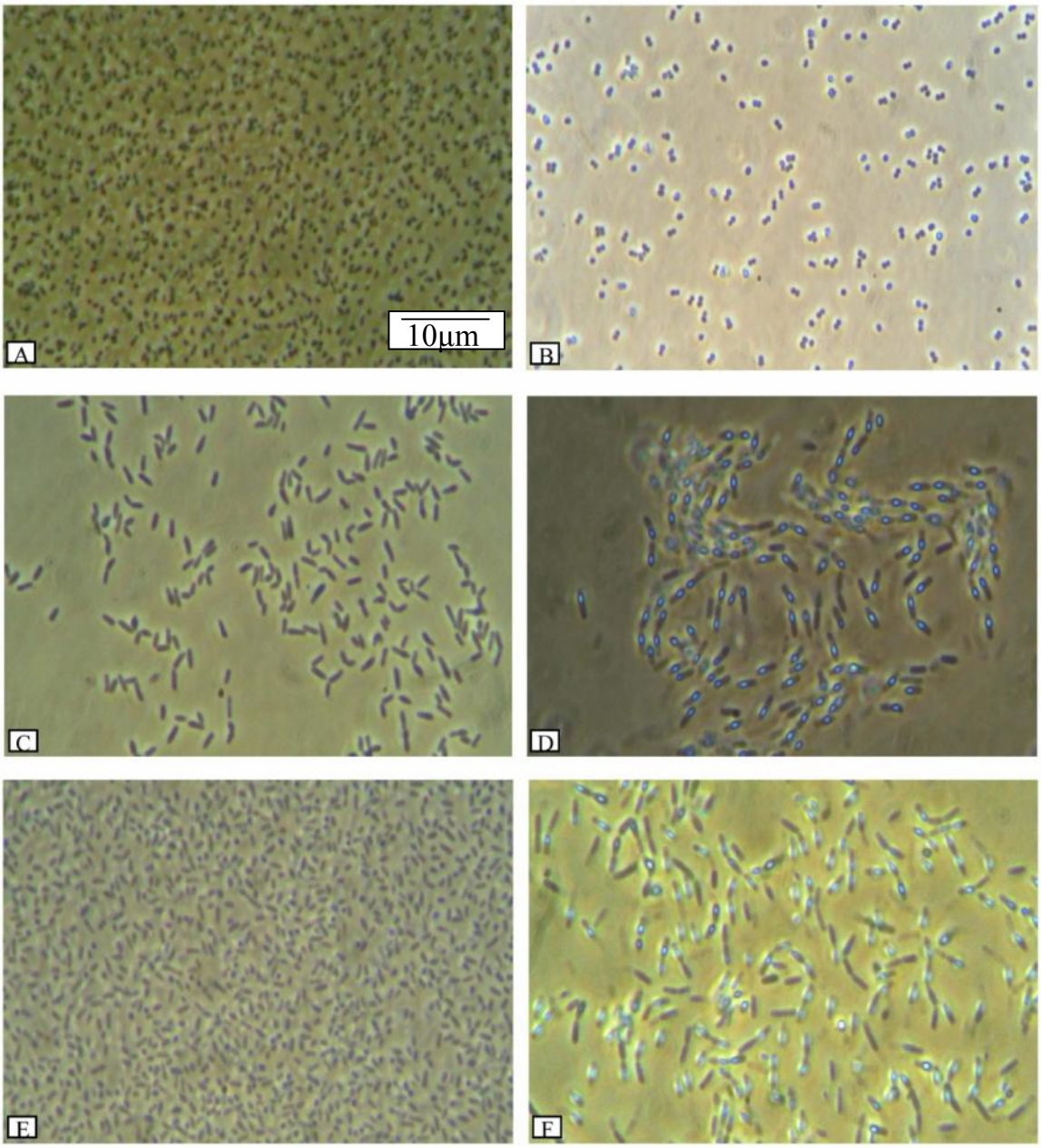


Plate. 3.4 Photomicrographs showing vegetative cells, spores and sporangia of the selected isolates under phase contrast microscope. A. S1/NAD1/4, B. S3/NA2/1, C. S3/MSA/2/1, D. S2/NA3/1, E. S3/TCBS1/1 and F. S2/EMB/2. Bar = 10µm.

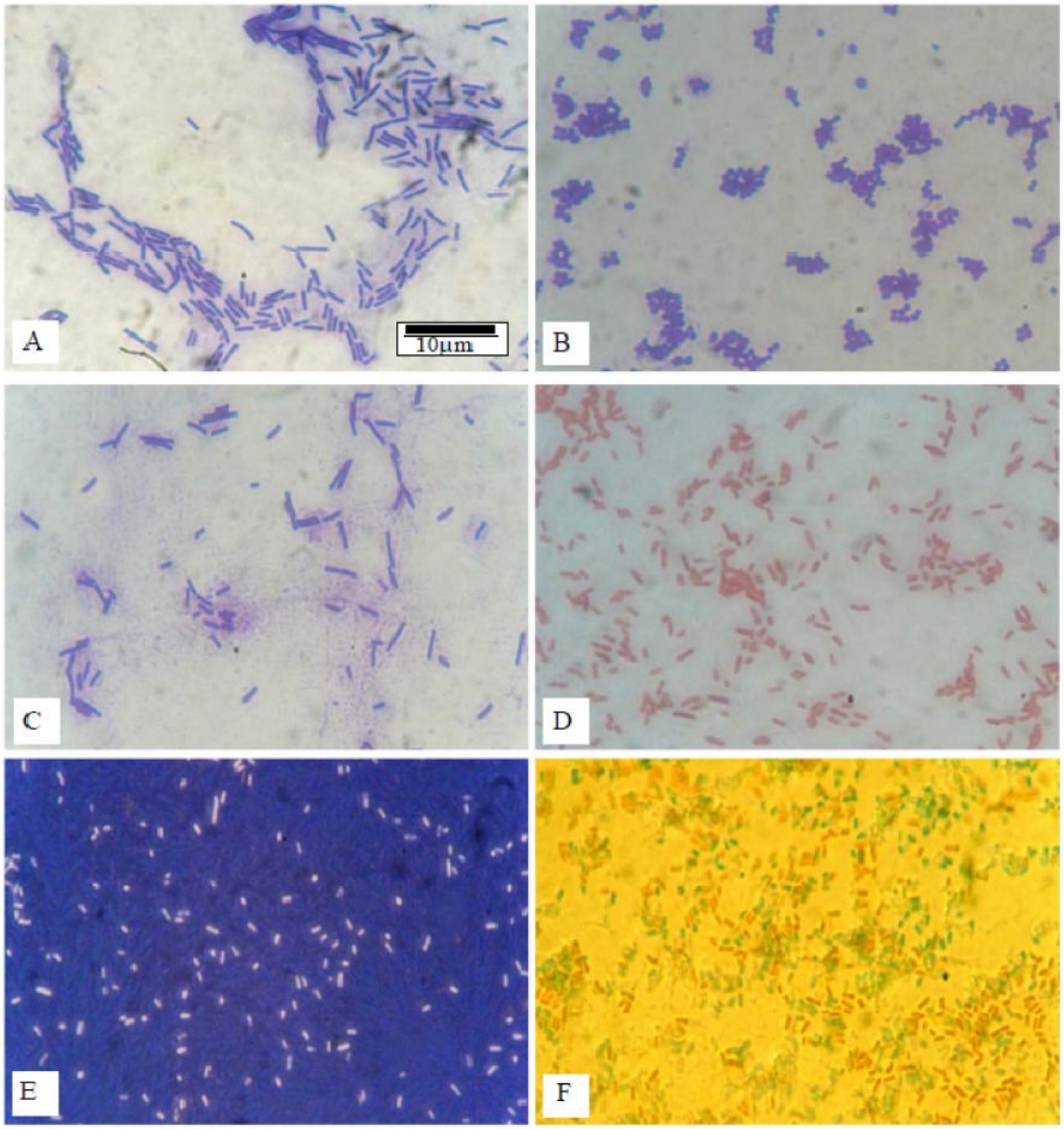


Plate. 3.5 Photomicrographs showing Simple staining of isolate no. A. S3/NA/3, B. S4/NAD2/1.Gram staining of C. S1/NAD1/3. & D. S3/EMB1/6 and Negative staining of E. S5/TCBS/1 and Spore staining of F. S3/NAD1/3. Bar = 10µm.

3.6 Physiological and biochemical characteristics of the selected isolates.

Major physiological and biochemical tests of the isolates were given in Table 3.9 to 3.11 and Fig. 3.5 to 3.20 and the test were grouped into two, *viz.* (1) tests for Gram positive bacteria and (2) tests for Gram negative bacteria.

3.6.1 Physiological and biochemical characteristics of Gram positive bacteria.

The result of the biochemical tests were given in Table 3.7. All the tested organisms were catalase positive. 8 isolates S1/NAD2′/1, S2/NAD2/1, S3/NA2/1, S2/MSA1/1, S2/MSA1'/1, S3/MSA1/1, S4/MSA1/2 and S5/MSA2/1 showed growth only at the surface of deep glucose agar medium and thus could be considered as strictly aerobes and other three isolates showed growth throughout the deep glucose agar medium thus could be considered as facultative anaerobes.

Among 12 Gram positive isolates 5 showed positive results for the V.P. test and 7 isolates showed positive results for M.R. test. Out of 11 Gram positive isolates only one isolate could produce gas from glucose. In case of casein hydrolysis three organisms S4/NAD2/1, S2/NAD3/1 and S6/NAD1/1 could hydrolyze casein. Only 3 isolates S4/NAD2/1, S3/NAD3/1, and S3/MSAD1/1 were able to hydrolyze starch. 5 of the isolated Gram-positive bacteria showed positive results for urease.

Out of the 12 isolates S6/NAD1/1, S2/MSA1/1, S4/MA2/1, could not utilize both citrate and propionate. 8 isolates could utilize citrate and propionate. Three organisms S6/NAD1/1, S4/TCBS1/1, S5/SS1/2 showed positive results for the oxidase test. Among the 12 tested organisms, S3/NAD2/1, S4/NAD2/1, S5/MSA1/1, could not reduce nitrate.

Table 3.9 Physiological and biochemical characteristics of the Gram positive isolates.

Isolate no.	Catalase	Deep glucose agar	Voges proskauer Test	Methtyl red	Gas from glucose	Casein	Starch	Citrate	Propionate	Phenylalanine	Nitrate reduction	Indole	Motility	Provisional identification
S2/NA/2/1	+	SA	-	+	-	-	-	+	+	-	-	-	-	*Micrococcus nishinomiyaensis*
S1/NA/2´/1	+	SA	-	-	-	-	-	-	+	-	-	-	+	*Planococcus citreus*.
S3/NA/2/1	+	SA	-	+	+	+	+	+	+	-	-	-	-	*Micrococcus varians*
S2/NA/3/1	+	FA	-	-	-	+	+	+	+	-	-	-	-	*Bacillus megaterium*
S5/NA/1/1	+	FA	+	+	-	+	-	-	-	-	+	-	-	*Staphylococcus lentus*
S2/MSA/1	+	SA	+	+	-	-	-	-	-	-	+	-	-	*Staphylococcus epidermidis*
S2/MSA/1´/1	+	SA	+	+	-	-	+	+	+	-	+	-	-	*Staphylococcus aureus*
S3/MSA/2/1	+	FA	+	-	-	-	-	-	-	-	-	-	+	*Listeria monocytogenes*
S3/MSA/1/1	+	SA	-	+	-	-	-	+	+	-	-	-	+	*Staphylococcuc aureus*
S4/MSA/1/2	+	SA	+	-	-	-	-	+	+	-	+	-	+	*Staphylococcus epidermidis*
S5/MSA/2/1	+	SA	+	+	-	-	-	+	+	-	+	-	-	*Staphylococcuc aureus*
S3/EMB/2/1	+	FA	+	-	-	+	+	+	+	-	-	+	-	*Bacillus marinus*

'+' sign indicates catalase activity positive ; Voges-Proskauer positive; Methyl red produced ; Gas not produced from glucose; Casein, Gelatin and Starch hydrolyzed; citrate and propionate utilized; Nitrate reduced; Indole produced; Tyrosine degraded; motile; lecithinase produced.

'-' Sign indicates, Voges-Proskauer negative; Methyl red negative; Gas not produced from glucose; casein, gelatin and starch not hydrolysed; citrate and propionate not utilized; Indole not formed; Tyrosine not degraded; No deamination of phenylalanine; non motile; no lecithinase produced;

SA=strict aerobes, FA= facultative anaerobes

3.6.2 Physiological and biochemical characteristics of Gram negative bacteria.

In fecal coliform test only S4/EMB2/1 and S6/EMB2/1 could produce both acid and gas whereas, isolates S4/EMB1/6, S6/EMB1'/1, S3/EMB2/2, S4/EMB1/4, S4/TCBS/1, S5/SS1/2, S4/SS2'/1 could produce acid from lactose. The Table 3.8 showed the results of fermentation tests of Gram negative isolates.

The results of physiological and biochemical tests presented in Table 3.9. All the organisms were catalase positive and showed positive result with the KOH solubility test. Among the 12 tested organisms only 5 isolates S3/EMB2'/1, S4/EMB1/4, S6/TCBS1/1 and S5/SSD1/2, S4/SSD2'/1 showed negative results in VP test. In case of MR test 6 isolates showed positive results.

In the hydrolysis test 2 Gram negative isolates showed positive results for casein hydrolysis.. The isolates S3/EMB2/2 and S4/TCBS/1 could hydrolyze starch. Seven isolates could utilize citrate and eight isolates could utilize propionate.
Only one isolate (S4/TCBS/1) could produce indole. Ten isolates were positive for nitrate reduction.

Out of 12 isolates 3 were positive to degrade urea. Two isolates (S4/TCBS/1 and S5/SS/1/2) could produce the enzyme oxidase. Only two organisms were able to produce H_2S in KIA test and 9 organisms produce gas. (Table 3.9).

Fig. 3.6, 3.7, 3.8, 3.9 and 3.10 showed the results (partial) of some important biochemical tests as carried out for the identification of the Gram positive and Gram negative bacteria.

Table 3.10 Physiological and biochemical characteristics of isolated (Gram-negative) strains.

Isolate No.	Catalase	KIA				MIU			MR	VP	Hydrolysis		Oxidase	KOH solubility	Deep glucose agar	Provisionally identified bacteria
		Slant	Butt	H_2S	Gas	Motility	Indole	Urease			Casein	Starch				
S2/EMB/2'/1	+	**K**	**A**	-	-	-	-	-	+	-	-	-	-	+	**SA**	*Proteus vulgaris*
S3/EMB/1/6	+	**K**	**A**	-	+	-	-	-	-	+	-	-	-	+	**FA**	*Salmonella paratyphi*
S3/EMB/1/2	+	**K**	**A**	-	+	+	-	+	-	+	-	-	-	+	**FA**	*Proteus morganii*
S5/EMB/1'/1	+	**K**	**A**	-	+	-	-	-	+	+	-	-	+	+	**SA**	*Salmonella paratyphi*
S5/EMB/2/1	+	**K**	**A**	-	+	+	-	+	-	+	-	-	-	+	**FA**	*Proteus morganii*
S2/EMB/2/2	+	**K**	**A**	-	+	-	-	-	-	+	+	+	-	+	**FA**	*Proteus vulgaris*
S3/EMB/1/4	+	**K**	**A**	-	+	+	-	+	+	-	-	-	-	+	**FA**	*Proteus morganii*
S3/TCBS/1	+	**K**	**K**	-	+	-	+	-	-	+	+	+	+	+	**SA**	*Vibrio sp.*
S5/TCBS/1	+	**K**	**A**	-	+	-	-	+	+	-	-	-	-	+	**FA**	*Proteus morganii*
S4/SS/1/2	+	**K**	**K**	+	-	+	-	+	+	-	-	-	+	+	**FA**	*Salmonella*
S3/SS/2/1	+	**K**	**A**	+	+	+	-	-	+	-	-	-	-	+	**FA**	*Salmonella*
S4/MSA/1/2	+	**N**	**N**	-	-	-	-	-	-	+	-	-	-	+	**SA**	*Halobacterium sp*

'+'sign indicates catalase activity positive, H_2S produced in KIA medium , Motile, Indole produced, urease produced, citrate utilized, Methyl red positive, VP positive, starch hydrolysed, oxidase activity positive, KOH solubility positive, lecithinase produced, A= acid (yellow), K= alkaline (red) reaction, SA= strict aerobes, FA= Facultative anaerobes, In case of proteolysis "+" indicates proteolytic activity.

Table 3.11 Acid production in the fermentation of the fecal coliform test by the isolated Gram negative isolates.

Isolates No	Lactose	
	Acid	Gas
S5/MSA/1/2	-	-
S3/EMB/2'/1	-	-
S4/EMB/1/6	+	-
S4/EMB/1/2	+	+
S6/EMB/1'/1	+	-
S6/EMB/2/1	+	+
S3/EMB/2/2	+	-
S4/EMB/1/4	+	-
S4/TCBS/1	+	-
S6/TCBS/1	-	-
S5/SS/1/2	+	-
S4/SS/2/1	+	-

'+' indicates acid and gas produced and '-' indicates acid and gas not produced

Plate. 3.6 Photographs showing fermentation of Lactose. Yellow color indicates positive reaction of lactose fermentation and green color indicates negative reaction**.**

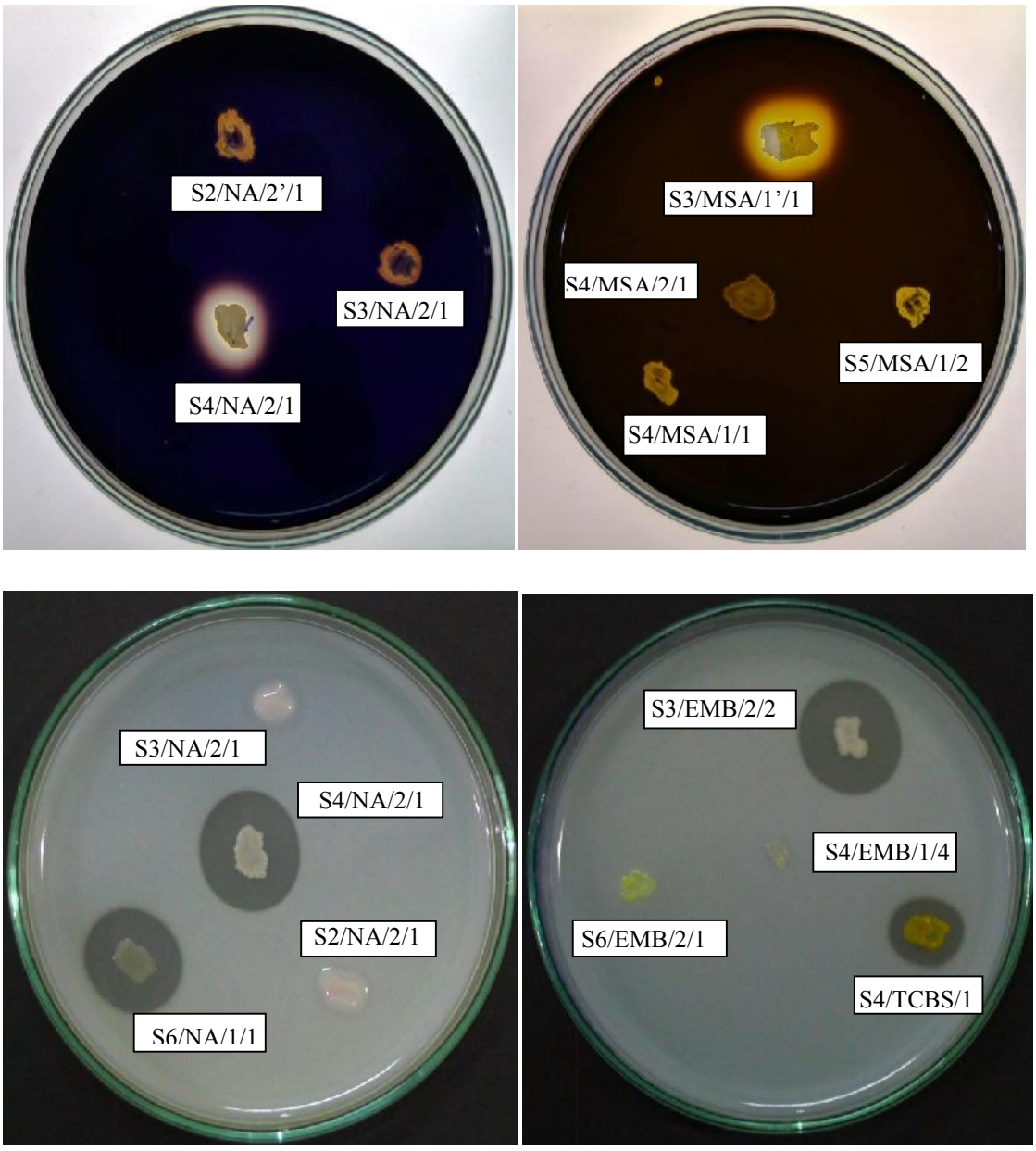


Plate. 3.7 Some biochemical tests of the selected isolates. A. hydrolysis of starch, C & D. Casein hydrolysis, the clear zone indicates positive reaction of hydrolysis of starch and casein.

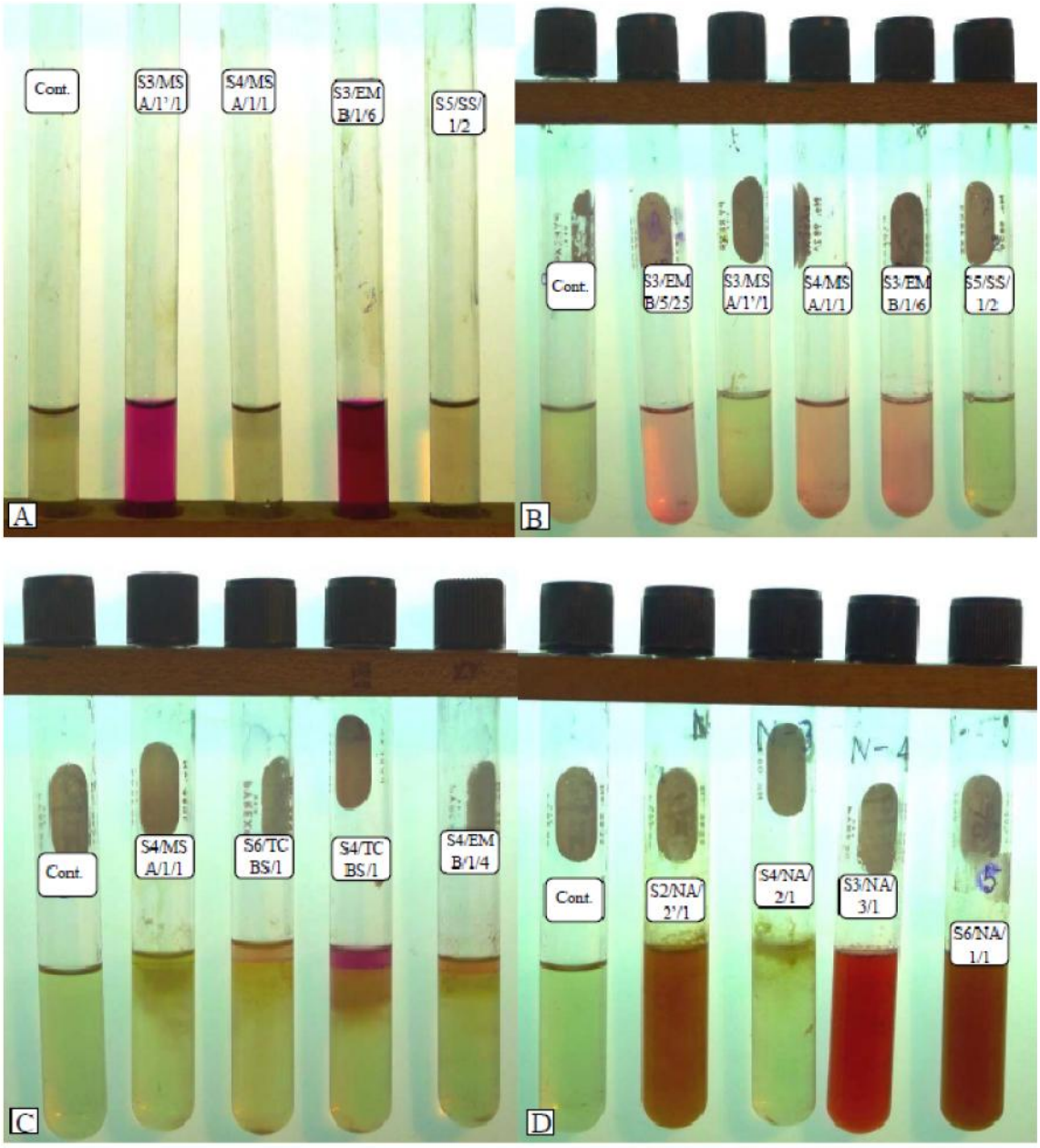


Plate. 3.8 Photographs showing some biochemical tests of the selected isolates. A. Voges Proskauer test, ruby red color indicates positive result**. B. Methyl Red test,** pink or red color indicates positive result**; C. Indole production, and D. Nitrate reduction test,** pink-red color indicates reduction of nitrate.

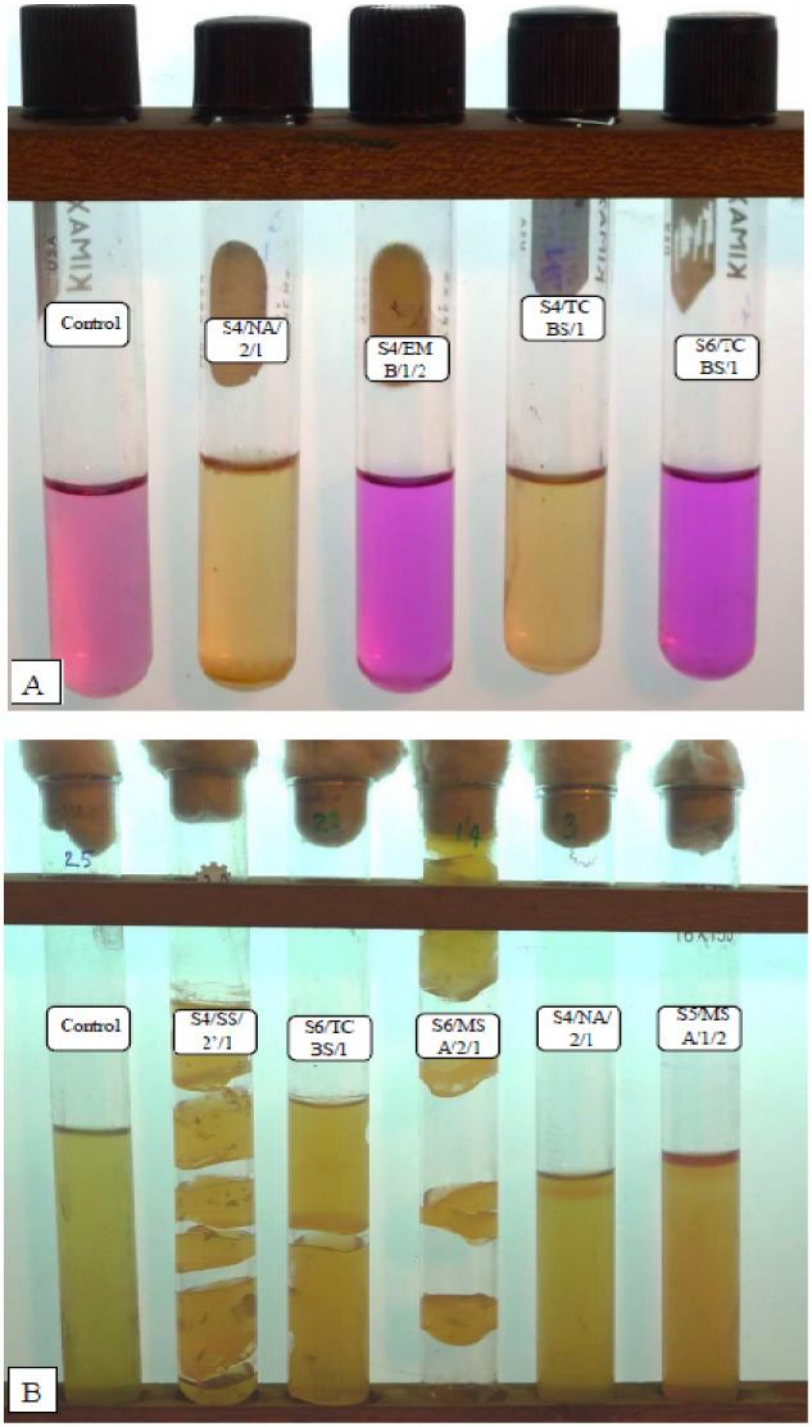


Plate.3.9 Photographs showing A. Urease production, Magenta color indicates positive result and **B. Deep glucose agar test,** growth on the agar surface indicates aerobic organisms, breakage of agar medium indicates production of gas from glucose and growth throughout the medium indicates the organisms are facultative anaerobes.

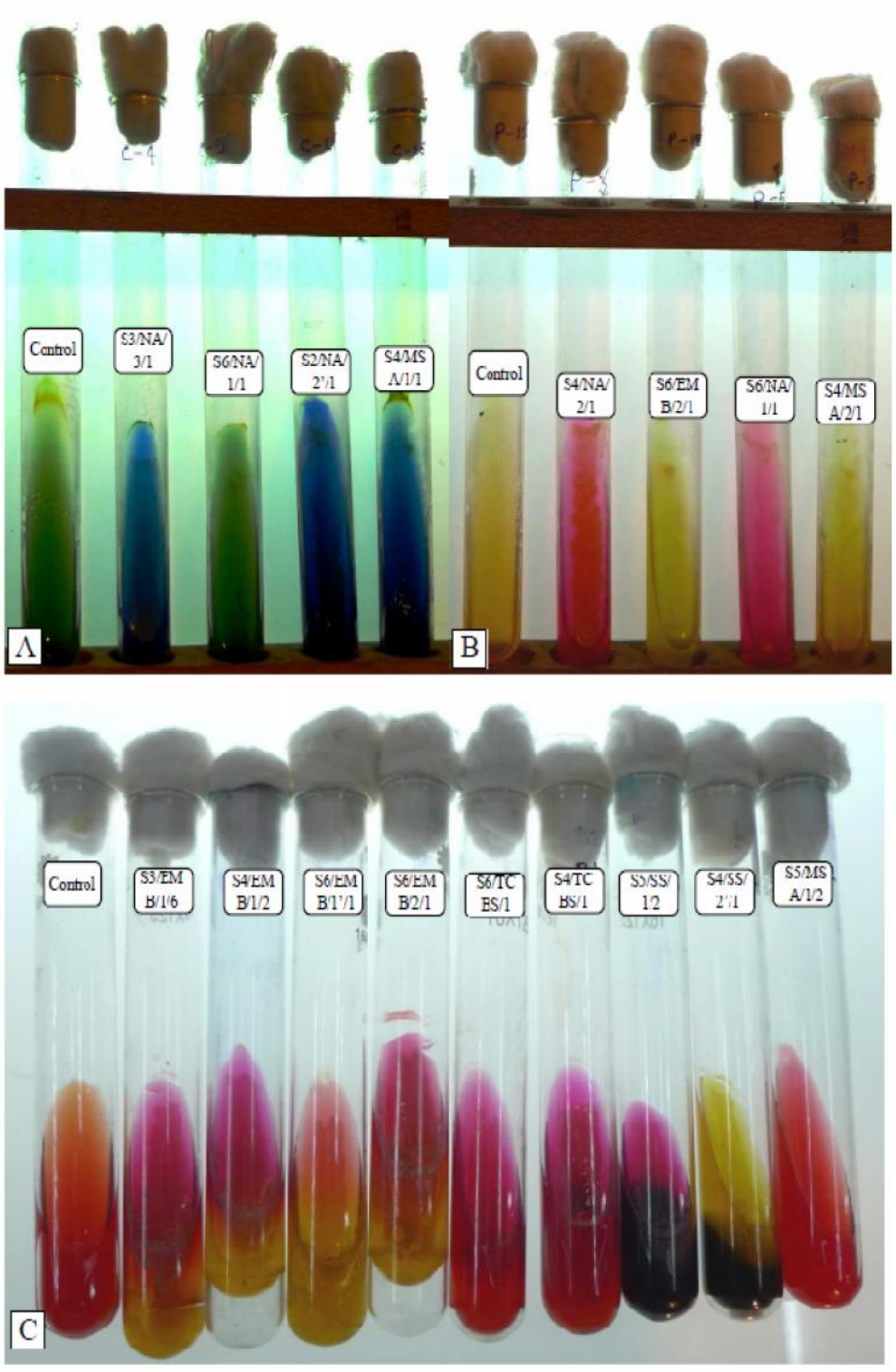


Plate. 3.10 Some biochemical tests of the selected isolates. A. Utilization of propionate, blue color indicates positive result**; B. Utilization of citrate,** pink color indicates positive result; **C. Kligler's Iron agar test,** yellow color indicates fermentation of suger, red color indicates not ferment suger and black color indicates production of H_2S gas.

3.7 Growth response at different concentrations of NaCl.

Effect of different salt concentrations (2, 4, 6, 8 and 10%) on the isolated bacteria was shown in Table 3.10 and Fig. 3.11 & 3.12. The results showed that the organisms had a wide range of tolerance towards NaCl concentration. The maximum growth of the organisms was in between 2-6% concentration of NaCl.

Table 3.12 Growth response expressed as OD at 600nm absorbance at different NaCl concentration

Isolate No.	NaCl concentrations (%)				
	2	4	6	8	10
S3/NA/2/1	0.499	0.498	0.372	0.189	0.152
S2/NA/2′/1	0.451	0.346	0.306	0.182	0.161
S4/NA/2/1	0.733	0.664	0.603	0.366	0.260
S3/NA/3/1	0.395	0.311	0.256	0.196	0.154
S6/NA/1/1	0.412	0.363	0.271	0.116	0.145
S3/MSA/1	0.658	0.556	0.482	0.411	0.352
S3/MSA/1′/1	0.582	0.489	0.456	0.392	0.489
S4/MSA/2/1	0.264	0.210	0.083	0.083	0.084
S4/MSA/1/1	0.561	0.571	0.431	0.278	0.249
S5/MSA/1/2	0.195	0.152	0.083	0.078	0.078
S6/MSA/2/1	0.800	0.782	0.613	0.504	0.545
S5/MSA/1/1	0.067	0.068	0.066	0.075	0.075
S3/EMB/2'/1	0.140	0.081	0.067	0.056	0.062
S3/EMB/2/1	0.323	0.259	0.257	0.178	0.199
S4/EMB/1/6	1.101	0.782	0.506	0.244	0.093
S4/EMB/1/2	0.773	0.558	0.388	0.148	0.107
S6/EMB/1'/1	0.293	0.513	0.92	0.056	0.062
S6/EMB/2/1	0.643	0.471	0.327	0.185	0.091
S3/EMB/2/2	0.263	0.271	0.236	0.096	0.088
S4/EMB/1/4	0.313	0.261	0.218	0.076	0.083
S4/TCBS/1	0.372	0.230	0.079	0.075	0.078
S6/TCBS/1	0.283	0.217	0.135	0.081	0.066
S5/SS/1/2	0.708	0.221	0.077	0.065	0.076
S4/SS/2/1	0.484	0.277	0.195	0.105	0.085

3.8 Provisional Identification of the isolates.

Consulting all observed and tested characters of the isolated organisms, identifications were done. The two separate groups (i) Gram positive, aerobic heterotrophic bacteria (ii) Gram-negative enteric and related bacteria were presented in Tables 3.13. For the purpose of identification Bergey's Manual of Systematic Bacteriology (Sneath *et al*. 1986) was followed for the aerobic heterotrophic bacteria while Manuals of WHO (1987), Bergey's Manual of Systematic Bacteriology (Krieg and Holt 1984) were used for Gram negative, enteric and related bacteria.

Table 3.13 Provisional identification of the bacteria isolated from collected samples.

S.I No	Type of sample	Isolate No.	Provisionally identified bacteria
1	Salted	S3/NA/2/1	*Micrococcus nishinomiyaensis*
2	Salted	S2/NA/2′/1	*Planococcus citreus*.
3	Fresh	S4/NA/2/1	*Micrococcus varians*
4	Salted	S3/NA/3/1	*Bacillus megaterium*
5	Fresh	S6/NA/1/1	*Staphylococcus lentus*
6	Salted	S3/MSA/1	*Staphylococcus epidermidis*
7	Salted	S3/MSA/1′/1	*Staphylococcus aureus*
8	Fresh	S4/MSA/2/1	*Listeria monocytogenes*
9	Fresh	S4/MSA/1/1	*Staphylococcuc aureus*
10	Fresh	S5/MSA/1/2	*Staphylococcus epidermidis*
11	Fresh	S6/MSA/2/1	*Staphylococcuc aureus*
12	Fresh	S5/MSA/1/1	*Halobacterium sp*
13	Salted	S3/EMB/2'/1	*Proteus vulgaris*
14	Salted	S3/EMB/2/1	*Bacillus marinus*
15	Fresh	S4/EMB/1/6	*Salmonella paratyphi*
16	Fresh	S4/EMB/1/2	*Proteus morganii*
17	Fresh	S6/EMB/1'/1	*Salmonella paratyphi*
18	Fresh	S6/EMB/2/1	*Proteus morganii*
19	Salted	S3/EMB/2/2	*Proteus vulgaris*
20	Fresh	S4/EMB/1/4	*Proteus morganii*
21	Fresh	S4/TCBS/1	*Vibrio sp.*
22	Fresh	S6/TCBS/1	*Proteus morganii*
23	Fresh	S5/SS/1/2	*Salmonella*
24	Fresh	S4/SS/2/1	*Salmonella*

3.9 Determination of Proximate Composition of Fish Sample

The major constituents in fish are water, protein, lipids and ash. Large fluctuations occur in the proximate composition and are influenced by several factors such as species of fish, diet, fishing grounds, season, sex and sexual maturity and spawning. Water content varies from 60-80%. Fatty fish exhibit an inverse relationship between fat and water. Protein content in most fish averages 18-20%, through general variation is in the range of 15-24%. Lipid content of fish muscle shows variation from as low as 0.2 to as high as 60-65%. Ash content varies between 0.4 and 4%..

The major component of fish muscle is moisture. The moisture content varied from 55.47 - 46.03% between dorsal to ventral portion. The highest value was found in dorsal portion of the fish whereas the lowest value was in ventral portion of the fish. This result more or less coincides with the findings of Mansur *et al*. in hilsa.

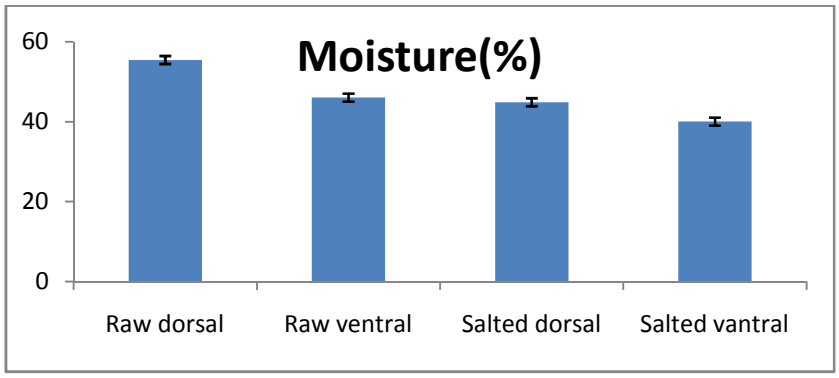


Fig .3.9.1. Variation of moisture contents (%) in different portions of fish. Moisture is significantly differed in the different portions of hilsa in different condition (ANOVA, HSD; $p < 0.05$) but do not differ significantly (ANOVA, HSD; $p > 0.05$

The protein content was estimated as 18.94, 24.25, 15.89 and 20.83% in dorsal portion (fresh sample), ventral portion (fresh sample), dorsal portion (salted sample) and ventral portion (salted sample) respectively (Fig. 4). The highest value was found in ventral portion (fresh sample) and lowest value recorded in ventral portion (fresh sample) of fish.

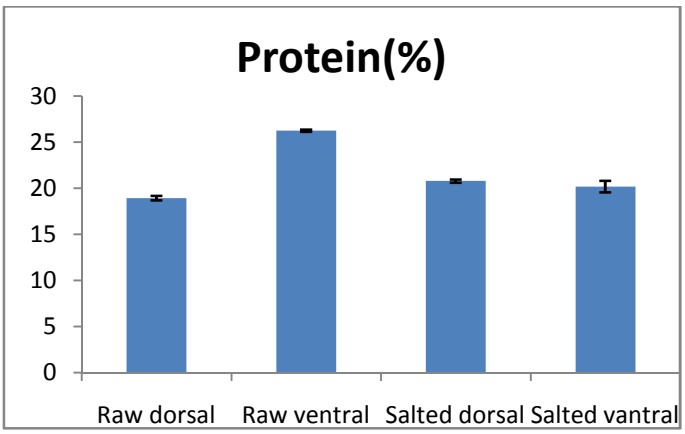


Fig 3.9.2. Variation of protein contents (%) in different portions of fish. Moisture is significantly differed in the different portions of hilsa in different condition (ANOVA, HSD; $p < 0.05$) but do not differ significantly (ANOVA, HSD; $p > 0.05$

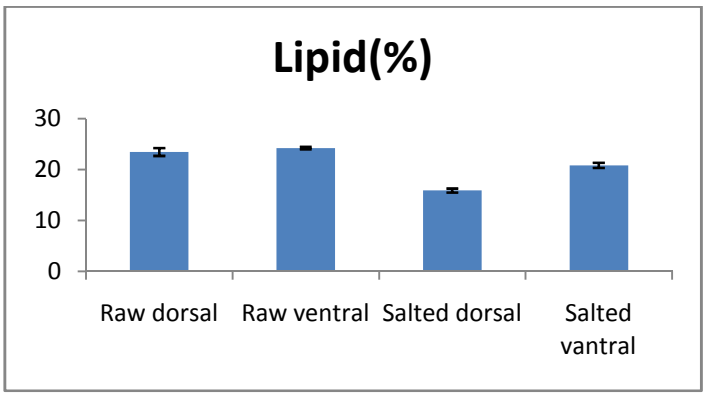


Fig 3.9.3. Variation of Fat contents (%) in different portions of fish. Moisture is significantly differed in the different portions of hilsa in different condition (ANOVA, HSD; $p < 0.05$) but do not differ significantly (ANOVA, HSD; $p > 0.05$

The fat contents in different portions of fish body in different condition is shown in Fig. 5. The highest value of fat content was recorded in ventral portion of fish body from the fresh sample (24.24%) and the lowest was in dorsal portion of fish body from the salted sample (15.89%). The fat content recorded 23.47, 24.24, 15.89 and 20.83% in dorsal portion (fresh sample), ventral portion (fresh sample), dorsal portion (salted sample) and ventral portion (salted sample) respectively, was similar to the result of Mansur *et al*. (8) in hilsa (24.87%) and might be due to habitat, season, age etc.

The highest ash content found in ventral portion of fish body from the salted fish (18.35%) and lowest in dorsal portion from the fresh sample (1.64%), Fig. 6). The value of ash in dorsal portion (fresh sample), ventral portion (fresh sample), dorsal portion

(salted sample) and ventral portion (salted sample) was recorded 1.64, 2.92, 18.15 and 18.35 respectively.

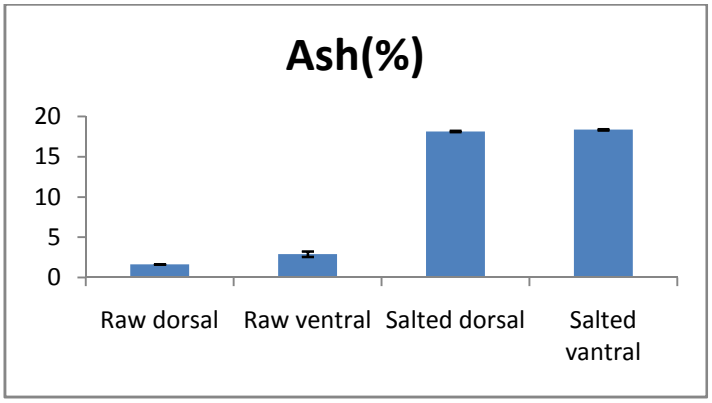


Fig .3.9.4. Variation of protein contents (%) in different portions of fish. Moisture is significantly differed in the different portions of hilsa in different condition (ANOVA, HSD; $p < 0.05$) but do not differ significantly (ANOVA, HSD; $p > 0.05$)

The result of the present investigation states the proximate composition of different portions of fish. However, variation in proximate composition of fish flesh may vary with different portions of fish body, species variation, season, age and the feeding habit of fish. The chemical composition of flesh may vary largely between and within species. In the present study the differences of proximate composition may be due to the difference in different portion of fish body used (ANOVA, HSD; $p < 0.05$).

4. DISCUSSION

Quality is the degree of excellence or grade of goodness. In simple terms, the quality of a food can be defined as those characteristics which make it acceptable to the consumers. For marketing of fish in fresh state, it is necessary to retain flavor, texture, odor and appearance. There are a number of parameters and standards (physical, chemical, microbial etc.) for the assessment of freshness quality of wet fresh fish. Bacteriological quality is of public health importance as it directly relates to spoilage of fish and becomes the cause of outbreak of food poisoning.

Raw fishes are highly perishable protein source that contain normal bacterial flora from their environments in addition to the contaminants occurred during harvesting and handling of the products. The living fishes carry populations of predominantly Gram negative psychrotrophic bacteria on their external skin, nearly 10^2-10^3 bacteria per gram. Coliform may be absent or present in very low density and *Salmonella, Shigella* and other enteric pathogens are usually not found as these are not he normal flora of fishes or of their environment (FAO 1979).

The processed food is considered as spoiled when the total bacterial count (TBC) values reach to 10^6 cfu/g or more in food items (Shewan 1970). The bacterial flora on newly caught fish depends on the environment rather than on the fish species (Shewan 1961). Another source of contamination of harmful microorganism could be fishing vessel (Waheb *et al*. 2003).

The total bacterial load in fresh fish was 11.8 x 10^7, 19.3 x 10^7 and 25 x 10^7cfu/g in muscle, skin and intestine respectively which is beyond the acceptable limit according to the ICMFS (ICMFS 1998 and FDA 2001). This might be due to contamination of source water from where the fishes were caught or might be due to secondary contamination during the time of handling as well as storage of fishes in ice made from contaminated water. According to (Hatha *et al*. 2003) high microbial abundance might be due to contaminated source of water, poor hygiene and sanitation condition of processing

All samples were observed having high quantity of total coliform exceeding the limit (>10^2 cfu/g) suggested by (ICMSF 1986) and proves local markets supply low quality fish. The presence of coliform confirms the sewage contagion. It also indicates the contamination during handling and selling process in markets including holding temperature. Moreover, the contamination may also come from the water use for washing or icing (Boyd 1990).

Bacterial and chemical decomposition have been reported to be the major factors contributing to the rapid deterioration of fish quality (Laycock 1971 and Morten *et al.* 2002) stated that the shelf life of fishery products is usually limited by microbial activity, although for some fatty fishes or at super chilled storage, it can be limited by non microbial activity.

Coliform bacteria are the indicator organisms whose presence in food in large quantity indicates the probability of having pathogenic bacteria. Faecal coliform are considered to be present especially in the gut and feces of warm-blooded animal. Because the origins of faecal coliforms are more specific than the origins of the total coliform group, faecal coliforms are considered a more accurate indication of animal or human waste than the total coliforms (CDC 2005). The presence of fecal coliform is not permitted in the shrimp samples in Japan, USA and other European countries (WHO 1995).

Salmonella in aquaculture fish products mainly originates from the environment rather than from poor standards of hygiene and sanitation. But sometimes, incidence of this bacterium in fish, shrimp or similar foods of aquatic habitats may be happened due to external contamination. Most fish are cooked prior to consumption. These products, therefore, cause negligible health risks to the consumers except for cross contamination in the kitchens (Huss 1995). *Salmonella* has been isolated from fresh, frozen, canned and sun dried marine fish products (Nataranjan *et al.* 1985). This bacterium is been isolated from different raw hilsa products in different market of Dhaka city during this study.

Vibrio was not found in a study of hilsa fish and indicated that this bacterium could survive less than tha of other bacteria associated with the fish (Shamsuzzaman 2011). Interestingly, in the present study *Vibrio* is present in only two samples collected from Kawran Bazar and

Ananda Bazar and it might be due to contamination during sell. *Vibrio* spp mainly are found in gut of the fishes.

The lack of proper knowledge, facilities and carelessness of the fish retailers about microbiological quality of the freshwater fish is evident from the study. It was also observed that the fish species collected from local market were not preserved in ice. These were kept open in normal temperature. So, after a certain period of time or after a few hours the fishes comes to the market from the catch point, the microbiological condition of fish become lower and the quality deteriorate. The result also indicates that the hygienic condition and sanitation facilities are not good in the markets of Dhaka city.

Five types of bacteriological culture media viz. nutrient agar (a generalized complex medium), EMB agar medium for coliform, SS agar for *Salmonella-Shigella* and MSA agar for *Staphylococcus* and TCBS for *Vibrio* were used to assess the quantitative and qualitative study. The bacterial load of salted fish samples was found to be ranged between 1.4×10^4 to 7.45×10^6 cfu/g, 6.4×10^3 to 7.75×10^4 cfu/g, 1.5×10^2 to 4.0×10^4 cfu/g on nutrient agar, EMB agar, and MSA agar, respectively. No bacterial colony was found on SS agar and TCBS agar plate in salted fish. On the other hand, the bacterial load of fresh fish was ranged between 6.6×10^4 cfu/g to 1.22×10^6 cfu/g, 1.5×10^2 to 6.35×10^3 cfu/g, 1.9×10^5 to 1.2×10^6 cfu/g and 2.5×10^2 to 1.64×10^5 on nutrient agar, SS agar, EMB agar and MSA agar respectively. The results clearly showed that fresh fish samples were loaded with high bacterial counts than salted fish.

Bacteria associated with fish muscle and their great variation in the percentage have been reported by(Anwar *et al.* 1988). The microbial contamination of the fish samples depend upon the source of the fish, pH, temperature, acidity of the water, natural handling, processing time elapsed between catching and marketing, water used during marketing etc. Depending on the habitat and other environment factors a wide range of variation in distribution of microflora in fish has been reported (Lakshmy 1999, Hess 1932 and Ito et al. 1993). It is important to note that when the total bacterial load reach $1.0x10^7$ cfu/g or more in food and food products, these foods are considered as spoiled and (Taylor 1920). The total viable bacterial count of salted Hilsa collected from different markets ranged from

$1.4x10^4$ to $7.45x10^4$ cfu/g, which means that the salted hilsa collected from some markets was not harmful for consumption.

According to WHO the presence of some organism such as *Bacillus megaterium*, *Bacillus subtilus*, *Micrococcus varians*, *Staphylococcus aureus* in large number in raw fish is harmful because of possible bacterial food poisoning. Presence of *Staphylococcus* sp. suggests higher level of environmental contamination and its presence indicates possible risk of food poisoning.

During this investigation a number of bacteria were isolated and finally 24 were selected for detail study. After thorough characterization with the available facilities the organisms were compared with the standard description in the Bergey's Manual of Systematic Bacteriology volume I and II (Sneath *et al.* 1986 and Krieg and Holt 1984) and on the basis of similarity, isolates were provisionally identified. Both Gram positive and Gram negative bacteria were found to be associated with the studied samples.

Among these isolates 12 were members of Gram positive bacteria. Out of these Gram positive members 9 isolates (S1/NAD1′/1, S1/NAD1/4, S8/NA1/5, S11/NAD1/3, S12/NAD1′/1, S12/NAD1/1, S12/NA1/1, S12/NA1/3) were cocci. Among them only one isolate (S2/NAD2′/1) *Planococcus,* six isolates S6/NA/1/1, S3/MSA/1, S3/MSA/1′/1, S4/MSA/1/1, S5/MSA/1/2, S6/MSA/2/1 were the member of *Streptococcus* and remaining two isolates were the member of *Micrococcus* (S3/NAD2/1 and S4/NAD3/1), respectively. Three isolates of Gram positive bacteria were rod shaped and two spore former of them fell under the genus *Bacillus*. The identified species were *Bacillus megaterium* and *B. marinus* and the remaining Gram positive non spore former rod was the member of *Listeria monocytogenes.*

According to (Huss *et al* 1995), Gram negative bacteria is more dominant over Gram positive bacteria in fish. However this depends on many factors such as fish species,

location of fish body from where sample is taken, storage time after dying, habitat of fish etc.

In the present study, it was found that salting can only reduce the growth of bacteria but it cannot prevent growth of bacteria.

The proximate composition of lona ilish, the mean values (as % muscle) of moisture, salt, and ash were found as 54.35, 15.75 and 16.73 respectively (Majumdar and Basu 2008). Food borne pathogenic bacteria are inhibited by a water activity of 0.92 or less that is equivalent to NaCl concentration of 13% (w/v) (FAO 1991). Possible explanation could be due to the preservative action of salt, that it exerts a toxic action; makes moisture unavailable for the microorganisms; prevents bacterial growth by dehydrating the cells by plasmolysis, and destroys bacterial protoplasm (Fabian *et al.* 1951). Salt content of dry salted mackerel and pink perch stored at ambient temperature were reported as 17.5% and >21.0%, respectively (Srikar *et al*. 1993). Salt content of salt fermented anchovy was found 19.40% (Hernandaz-Herrero *et al.* 1999). Salt content of lona ilish was found to be less than most of the other fermented fish products which is significant from the view point that high dietary salt pose a severe health risk. The pH and total titratable acidity (TTA) of lona ilish were found as 5.66% and 0.98%. The reason of law pH value of the product may be attributed to the fact that samples were undergoing fermentation and not spoilage.

Post-harvest loss was estimated in 2 types of salted products, i. dry salted hilsa and ii. wet salted hilsa (Nowsad 2010). In both cases, 60% of the raw material lost their quality. Pre-process (handling, washing) and in-process (dressing, cutting, salting, piling, etc.) losses ranged between 5-7%. Quality losses in final products were 65.6% and 67% respectively in dry and wet salted hilsa. Smoked hilsa contain 39.65% moisture, 25.65% protein, 24.85% fat, 3.5% ash and 16.2% salt (Hossain *et al.* 2012).

The major component of fish muscle is moisture. The moisture content varied from 54.69 - 56.59% .The highest value was found in dorsal portion of the fish whereas the lowest value was in ventral portion of the fish (Shamim *et al* 2011).

The major component of fish muscle is moisture. The moisture content varied from 55.47 - 46.03% between dorsal to ventral portion. The highest value was found in dorsal portion of the fish whereas the lowest value was in ventral portion of the fish. This result more or less coincides with the findings of (Mansur *et al* 1998) in hilsa

The protein content was estimated as 18.94, 24.25, 15.89 and 20.83% in dorsal portion (fresh sample), ventral portion (fresh sample), dorsal portion (salted sample) and ventral portion (salted sample) respectively (Fig. 4). The highest value was found in ventral portion (fresh sample) and lowest value recorded in ventral portion (fresh sample) of fish.

The fat contents in different portions of fish body in different condition is shown in Fig. 5. The highest value of fat content was recorded in ventral portion of fish body from the fresh sample (24.24%) and the lowest was in dorsal portion of fish body from the salted sample (15.89%). The fat content recorded 23.47, 24.24, 15.89 and 20.83% in dorsal portion (raw sample), ventral portion (fresh sample), dorsal portion (salted sample) and ventral portion (salted sample) respectively, was similar to the result of (Mansur *et al*.1998) (8) in hilsa (24.87%) and might be due to habitat, season, age etc.

The highest ash content found in ventral portion of fish body from the salted fish (18.35%) and lowest in dorsal portion from the raw sample (1.64%), Fig. 6). The value of ash in dorsal portion (fresh sample), ventral portion (fresh sample), dorsal portion (salted sample) and ventral portion (salted sample) was recorded 1.64, 2.92, 18.15 and 18.35 respectively.

The result of the present investigation states the proximate composition of different portions of fish. However, variation in proximate composition of fish flesh may vary with different portions of fish body, species variation, season, age and the feeding habit of fish. The chemical composition of flesh may vary largely between and within species.

CONCLUSION

Fresh water fish in Bangladesh are playing a significant role in the national economy. *Tenualosa ilisha* is very rich in nutritional properties and our national fish. Several million of people in Bangladesh are directly or indirectly involved with fish farming. Since fish is the main source of animal protein and *T. ilisha* is one of the sources of animal protein.

Traditionally, the fishermen themselves process the fish for salted hilsa. Hygienic conditions are far from satisfactory in the manufacturing units. Sometimes fish are not even washed before being cut. Secondly, the crude salt used is of cheap and unknown quality. Quality of the product largely depends on the freshness of the raw fish, removal of water during dry salting, period of maturation, concentration of brine etc.

It may be concluded that bacterial load in salted hilsa is less compare to that of raw hilsa in different market of Dhaka city. Moreover, there was no Salmonella in the salted hilsa. From the study, it can be concluded that hisa can be preserved by proper salting process. Regular microbial monitoring of fish market should be done to avoid microbial contamination of the fish during sell. Besides monitoring consumer's awareness should be increased through awareness building program about microbiological and chemical status of the fishes of our country. The study revealed that assessing the microbiological quality of fishes have great potential for further research and more importance should given on the microbiological quality assurance.

Recommendations

1. Current study was conducted only in very short periods, so further studies are to be needed for better understanding of microorganisms associated with the fish samples.
2. During processing of fishes the microbial quality of the products should be monitored and hazard should be controlled in each steps of processing.
3. Cross check in the experiment should be done to establish the pre-treatment process for people by indigenous materials for long time preservation.

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Appendix

Composition of the media and reagents used in this study are as follows.

1. Ammonium Crystal violet solution (SAB 1957)

Solution A

Crystal violet (85% dye content)	2.0 g
Ethyl alcohol (95%)	20 ml

Solution B

Ammonium oxalate	0.8 g
Distilled water	80 ml

Solution A and B were mixed, Stored for 24 hrs before use.

2. Basal medium for fermentation (SAB 1957)

Beef extract	3.0 g
Amonium dihydrogen phosphate	1.0 g
Magnesium sulphate	0.2 g
Potassium chloride	0.2 g
Carbohydrate	10.0 g
Bromothymol blue	2.0 ml
Distilled water	1000 ml

3. Congo red solution (SAB 1957)

Congo red (80% dye content)	2.0 gm
Distilled water	100 ml

4. Deep glucose agar medium (Hall 1929)

Beef extract	3.0 gm
Peptone	5.0 gm
Glucose	10.0 gm
Agar	15.0 gm
Distilled water	1000 ml

5.Eosin Methylene Blue (EMB) agar medium (Scharlau Chemie S.A)

Dehydrated form

6. Ferric chloride solution (Sneath *et al*. 1986)

$FeCl_3$	10 g
Distilled water	100 ml

7. Indole nitrate broth (Atlas 1997)

Tryptone	10.0 g
Distilled Water	1000ml
pH	7.2

8. Iodine solution (SAB 1957)

Iodine	0.33 g
Potassium Iodide	0.66 g
Distilled water	100 ml

9. Kligler's Iron Agar (KIA) Medium (Atlas 1997)

Peptone	20.0 g
Lactose	10.0 g
Glucose	1.0 g
NaCl	5.0 g
Ferric citrate	0.3 g
Beef extract	3.0 g
Yeast extract	3.0 g
$Na_2S_2O_3$	0.3 g
Agar	12.0 g
Phenol red	0.05 g
Distilled water	1000 ml
pH	7.4 ± 0.2 at 25°C

10. KOH-creatine solution (SAB 1957)

KOH	40.0 g
Creatinine	0.3 g
Distilled water	100 ml

11. Kovac's reagent (SAB 1957)

Para-dimethyl-amino-benzaldehyde	5.0 g
Butyl alcohol	75 ml
HCI (Conc.)	25 ml

12. Lactose Broth (Biolife Manual 1991)

Beef extract	3 g
Peptone	5 g
Lactose	5 g
Bromo-thymol-blue	(1.6% alcoholic solution)
Distilled water	1000 ml
pH	6.8

13. Malachite green solution (Claus 1995)

Malachite green	5.0 g
Distilled water	100 ml

14. Manitol salt agar (MSA) medium (Scharlau Chemie S.A)

Dehydrated form

15. Mercurochrome solution (SAB 1957)

Mercurochrome	0.5 gm
Distilled water	100 ml

16. Methyl Red/Voges-Proskauer broth medium (Sneath *et al.* 1986)

Protease peptone	7.0 gm
Glucose	5.0 gm
NaCl	5.0 gm
Distilled water	1000 ml

17. Methyl red solution (Bryan 1950)

Methyl red	0.1 gm
Ethyl alcohol (95%)	300 ml
Distilled water	200 ml

18. α-Napthol solution (Bryan 1950)

α –Napthol	l5.0 g
Ethyl alcohol (95%)	100ml

19. Nitrate broth medium (SAB 1957)

Peptone	5.0 g
Beef extract	3.0 g
NaCl	5.0 g
Potassium nitrate	1.0 g
Distilled water	1000ml

20. Nutrient agar medium (Pelczar 1993)

Beef extract	3.0 g
Peptone	5.0 g
NaCl	5.0 g
Agar	15.0 g
Distilled water	1000ml

21. Nutrient broth medium (Pelczar 1993)

Beef extract	3.0 g
Peptone	5.0 g
NaCl	5.0 g
Distilled water	1000 ml

22. Oxidase test reagent (Claus 1995)

Tetramethyl-p-phenylene-diamine dihydro-chloride	1.0 g
Distilled water	100 ml

23. Peptone broth

Peptone	10.0 g
Distilled water	1000 ml

24. Physiological saline

Sodium chloride	0.85 g
Distilled water	100 ml

25. Propionate agar medium (Sneath *et al*. 1986)

Sodium propionate	2.0 g
MgSO4.7H2O	1.2 g
$(NH_4)_2$ HPO4	0.5 g
KCI	1.0 g
Trace element solution	40.0 ml
Agar	15 g
Distilled water	920 ml
Phenol red (0.04% w/v)	20.0 ml
pH	6.8

26. Saffranin solution (SAB 1957)

Safranin	0.5 g
Distilled water	100 ml

27. Simmon's citrate agar (Atlas 1997)

$MgSO_4$. $7H_2O$	0.2 g
Mono-ammonium phosphate	1.0 g
Di-Potasssium phosphate	1.0 g
Na-citrate	2.0 g
NaCI	5.0 g
Bromo-thymol-blue	0.08 g
Agar	15.0 g
Distilled water	1000 ml.
pH	6.9 ± 0.2 at 25°C

28. Skim Milk Agar (SMA) medium (Collins and Lyne 1984)

Skim Milk	6.6 ml
Nutrient agar	100 ml
pH	7.2

29. SS agar (Atlas 1997)

Agar	12.0g
Lactose	10.0g
Sodium citrate	10.0g
$Na_2S_2O_3$	8.5g
Bile salts	5.5g
Beef extract	5.0g
Peptone	5.0g
Ferric citrate	1.0g
Neutral red	0.025g
Brilliant green	0.33mg
pH	7.3

30. Starch agar medium (Claus 1995)

Beef extract	3.0 g
Peptone	5.0 g
Soluble starch	10.0 g
Agar	5.0 g
Distilled water	1000 ml

31. TCBS agar medium (Scharlau Chemie S.A)

Dehydrated form

32. Trace element solution (Sneath *et al.* 1986)

EDTA	500.0 mg
$FeSO_4.7H_2O$	200.0 mg
$ZnSO_4.7H_2O$	10.0 mg
$MnCl_2.4H_2O$	3.0 mg
H_3BO_3	30.0 mg
$CoCl_2.6H_2O$	20.0 mg
$CuCl_2.2H_2O$	1.0 mg.
$NiCl_2.6H_2O$	2.0 mg
$Na_2MoO_4.2H_2O$	3.0 mg
Distilled water	1000 ml

*40 ml trace eliment solution was added to the medium.

33. Urease broth (Rustigen & Stuart 1941)

K_2HPO_4	9.1 g
Na_2HPO_4	5 g
Yeast extract	0.1 g
Phenol red	0.01 g
Distilled water	1000 ml
pH	7.2

10 ml of a 15 % (w/v) solution of urea sterilized by filtration was added to 75 ml of sterile urease broth.

Total bacterial count

	Type of fish	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	Salted of Palashi Bazar	2	14000.0000	
	Raw of New Market	2	66000.0000	
	Salted of Anando Bazar	2	68500.0000	
	Salted of New Market	2	70000.0000	
	Salted of Kawran Bazar	2	74500.0000	
	Raw Ananda Bazar	2		930000.0000
	Raw of Palashi Bazar	2		980000.0000
	Raw Kawran Bazar	2		1220000.0000
	Sig.		.999	.353

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

EMB count

	Type of fish	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	Salted of Palashi Bazar	2	5200.0000		
	Salted of Anando Bazar	2	6400.0000		
	Salted of Kawran Bazar	2	8500.0000		
	Salted of New Market	2	77500.0000	77500.0000	
	Raw of New Market	2	190000.0000	190000.0000	
	Raw of Palashi Bazar	2	215000.0000	215000.0000	
	Raw Ananda Bazar	2		266000.0000	
	Raw Kawran Bazar	2			1200000.0000
	Sig.		.092	.144	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

MSA count

	Type of fish	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	Salted of New Market	2	150.0000	
	Raw of New Market	2	250.0000	
	Salted of Palashi Bazar	2	2100.0000	
	Salted of Anando Bazar	2	3200.0000	
	Salted of Kawran Bazar	2	40000.0000	
	Raw of Palashi Bazar	2	51000.0000	
	Raw Kawran Bazar	2		126000.0000
	Raw Ananda Bazar	2		164000.0000
	Sig.		.087	.267

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Salmonella /shigella

	Type of fish	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	Raw of New Market	2	.0000	
	Salted of Palashi Bazar	2	.0000	
	Salted of Kawran Bazar	2	.0000	
	Salted of Anando Bazar	2	.0000	
	Salted of New Market	2	.0000	
	Raw Ananda Bazar	2	150.0000	
	Raw of Palashi Bazar	2		4900.0000
	Raw Kawran Bazar	2		6350.0000
	Sig.		1.000	.708

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Total vibrio count

	Type of fish	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	Raw Ananda Bazar	2	.0000
	Raw of New Market	2	.0000
	Salted of Palashi Bazar	2	.0000
	Salted of Kawran Bazar	2	.0000
	Salted of Anando Bazar	2	.0000
	Salted of New Market	2	.0000
	Raw of Palashi Bazar	2	50.0000
	Raw Kawran Bazar	2	50.0000
	Sig.		.829

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

SPSS output of the bacterial load analyses

Case Summaries[a]

				Total bacterial count	EMB count	MSA count	Salmonella /shigella	Total vibrio count
Type of fish	Raw of Palashi Bazar	1		860000,00	200000,00	62000,00	3800,00	,00
		2		1100000,00	230000,00	40000,00	6000,00	100,00
			N	2	2	2	2	2
		Total	Mean	980000,0000	215000,0000	51000,0000	4900,0000	50,0000
			Std. Error of Mean	120000,00000	15000,00000	11000,00000	1100,00000	50,00000
	Raw Kawran Bazar	1		1340000,00	1300000,00	132000,00	7700,00	,00
		2		1100000,00	1100000,00	120000,00	5000,00	100,00
			N	2	2	2	2	2
		Total	Mean	1220000,0000	1200000,0000	126000,0000	6350,0000	50,0000
			Std. Error of Mean	120000,00000	100000,00000	6000,00000	1350,00000	50,00000
	Raw Anand Bazar	1		760000,00	217000,00	188000,00	100,00	,00
		2		1100000,00	315000,00	140000,00	200,00	,00
			N	2	2	2	2	2
		Total	Mean	930000,0000	266000,0000	164000,0000	150,0000	,0000
			Std. Error of Mean	170000,00000	49000,00000	24000,00000	50,00000	,00000
	Raw of New Market	1		68000,00	150000,00	300,00	,00	,00
		2		64000,00	230000,00	200,00	,00	,00
			N	2	2	2	2	2
		Total	Mean	66000,0000	190000,0000	250,0000	,0000	,0000
			Std. Error of Mean	2000,00000	40000,00000	50,00000	,00000	,00000

	1		8000,00	4900,00	2300,00	,00	,00
	2		20000,00	5500,00	1900,00	,00	,00
Salted of Palashi Bazar		N	2	2	2	2	2
	Total	Mean	14000,0000	5200,0000	2100,0000	,0000	,0000
		Std. Error of Mean	6000,00000	300,00000	200,00000	,00000	,00000
	1		80000,00	7000,00	30000,00	,00	,00
	2		69000,00	10000,00	50000,00	,00	,00
Salted of Kawran Bazar		N	2	2	2	2	2
	Total	Mean	74500,0000	8500,0000	40000,0000	,0000	,0000
		Std. Error of Mean	5500,00000	1500,00000	10000,00000	,00000	,00000
	1		37000,00	6800,00	2400,00	,00	,00
	2		100000,00	6000,00	4000,00	,00	,00
Salted of Anand Bazar		N	2	2	2	2	2
	Total	Mean	68500,0000	6400,0000	3200,0000	,0000	,0000
		Std. Error of Mean	31500,00000	400,00000	800,00000	,00000	,00000
	1		80000,00	60000,00	100,00	,00	,00
	2		60000,00	95000,00	200,00	,00	,00
Salted of New Market		N	2	2	2	2	2
	Total	Mean	70000,0000	77500,0000	150,0000	,0000	,0000
		Std. Error of Mean	10000,00000	17500,00000	50,00000	,00000	,00000
	N		16	16	16	16	16
Total	Mean		427875,0000	246075,0000	48337,5000	1425,0000	12,5000
	Std. Error of Mean		126742,54991	97011,12501	15580,25159	652,84633	8,53913

a. Limited to first 100 cases.

SPSS output of the proximate composition analyses

Case Summaries[a]

				moisture	protein	fat	ash
fishtype	Raw DORSAL	1		55,49	19,82	22,17	1,62
		2		55,76	19,50	22,11	1,66
		3		55,39	18,13	24,64	1,61
		4		55,23	18,32	24,96	1,67
		Total	N	4	4	4	4
			Mean	55,4675	18,9425	23,4700	1,6400
			Std. Error of Mean	,11123	,42116	,77075	,01472
	Raw VENTRAL	1		45,38	26,25	24,72	2,32
		2		45,90	26,50	24,43	2,43
		3		46,49	26,31	23,70	3,78
		4		46,34	25,95	24,12	3,14
		Total	N	4	4	4	4
			Mean	46,0275	26,2525	24,2425	2,9175
			Std. Error of Mean	,24951	,11404	,21842	,34011
	salted dorsal	1		44,29	21,20	16,03	17,89
		2		44,23	20,92	16,96	18,31
		3		45,50	20,65	15,38	18,17
		4		45,50	20,40	15,20	18,24
		Total	N	4	4	4	4
			Mean	44,8798	20,7925	15,8925	18,1525

		Std. Error of Mean	,35802	,17240	,39798	,09205
	1		40,10	19,01	21,51	18,45
	2		40,35	19,20	21,85	18,10
	3		39,83	21,10	20,05	18,41
salted ventral	4		39,90	21,46	19,90	18,43
		N	4	4	4	4
	Total	Mean	40,0450	20,1925	20,8275	18,3475
		Std. Error of Mean	,11665	,63334	,49800	,08290
	N		16	16	16	16
Total	Mean		46,6049	21,5450	21,1081	10,2644
	Std. Error of Mean		1,44647	,74378	,87396	2,06684

a. Limited to first 100 cases.