CHAPTER I

INTRODUCTION

A. General Background

Fishes are the important members of animal kingdom belonging to the class Pisces. They form one of the most important groups of vertebrates for man, influencing his life in various ways (Khanna, 1970). They are among the earliest vertebrates, as early as Devonian period (350 million years back), popularly known as the Age of Fishes. About 30,000 species of fishes are known, some of, which are cosmopolitan in distribution. Some live in fresh water, many are marine; others live partly in fresh and partly in marine waters, while still others prefer brackish water (Anonymous, 1962).

Knowledge on the fish of Nepal dates back to the early 19th century. Hamilton was the first author to provide valuable information about the fish of Nepal. He described some fishes of Nepal in his admirable works Fishes of Ganges in the year 1822. The Nepal Himalaya is well-known for its fresh water fish (Shrestha, 1991). There are 184 fish species in Nepal belonging to 93 genera, 31 families and 11 orders (Shrestha, 1995, 2001 and 2003).

Man has been consuming fish since time unknown. About 75 percent of the world fish catch is used for human consumption. The per capita consumption rate of fish is 2 kg per year in Nepal and 5 kg per year in India and more than 8 kg in developed countries (K.C.1999).

Global production from capture fisheries and aquaculture supplied about 101 million tonnes of food fish in 2002, providing an apparent per capita supply of 16.2 kg (live weight equivalent), with aquaculture accounting for the growth in per capita supply since 2000. Overall, fish provided more than 2.6 billion people with at least 20 percent of their average per capita animal protein intake (Anonymous, 2004). Asia, which combines a relatively high per capita consumption with large populations, is by far the most important fish-consuming region. Europe is the second largest food fish-consuming continent. Fish consumption is generally higher in developed countries than in developing countries, notable exceptions being found among the

small developing island states. The lowest levels of consumption occur in Africa and the Near East (Anonymous, 1998).

In many countries, especially developing countries, the average per capita consumption may be low, but fish may be the staple food in coastal areas and among the poor, and an important source of animal protein. The demand for fish for food is expected to continue to grow. Based on projected population growth and on the maintenance of the present world level of consumption, by 2010 it could reach 120 million tonnes a year, a substantial increase over the 75 million to 85 million tonnes of the mid-1990s (Anonymous, 1998).

Information on fish demand and consumption in Nepal is very limited. Fish is generally accepted and liked by all non-vegetarians in Kathmandu. As up to 75% of the fish sold in the Kathmandu market come from India they are the lowland, warm water fish species. The majority of marketed fish are the major Indian carps Rohu (50%) and Katla (20%), and the Chinese silver carp (15%); the rest are other fish species (Dahal, 1998). Hill stream fish such as *Tor*, *Neolissocheilus*, *Schizothorax*, *Glyptothorax* and *Garra* are sold in smoked form in Kathmandu market, where they are brought from adjacent areas such as Trisuli, Dolalghat and Panauti (Shrestha, 1994).

About 10 million people die every year in the world either by starvation or by malnutrition. Absence of protein or inadequate supply of protein in diet is one of the major factors causing malnutrition. Fish contribute a significant amount of animal protein to the diets of people worldwide. It is estimated that between 15 and 20 percent of all animal proteins come from aquatic animals. Fish is highly nutritious and serves as a valuable supplement in diets lacking essential vitamins and minerals (Srivastava, 1985).

Thus the consumption of fish and other products from fishing can provide an excellent means of combating protein-calorie malnutrition, especially when the diet is based on starchy products very poor in protein. Fish in fact contains 15% to 20% of its dry weight as protein which is easily assimilated, as well as vitamins A, B and D, minerals (calcium, phosphorous and iron) and trace elements (fluorine and iodine in sea fish). Some fish species contain little fat, while others contain up to 20% lipids. The frequency of vitamin A deficiency, goiter and nutritional anemia could fall substantially if the consumption of fish were increased (Sasson, 1990).

Carbohydrates are present in negligible quantities in fish flesh. Glycogen is present in living fish and is rapidly converted to lactic acid after death. Thus fish flesh is often recommended in diet to those people who are willing to loose weight. Fish muscle also contains appreciable quantities of proteolytic enzymes. Amylase, lipase, thiaminase, adenosine triphosphates, choline esterase and other esterases, nuclease, butyrinase and glycogenase are reported to be present. Thiaminase occurs in freshwater fishes, but not in marine species (Anonymous 1962). .

Oils derived from fish can be classified broadly into two types: body oils expressed from whole fish, and liver oils extracted only from livers. Liver oils are of medicinal value and used primarily as sources of vitamin A. Body oils find use for edible and industrial purposes. Generally the species whose flesh is rich in oil possess livers with low oil content and vice-versa (Anonymous 1962).

However, there has been a very little work in Nepal regarding the study of protein, fat, minerals and vitamin and other contents of the freshwater fish. Most of the above data are from Indian fish species, both freshwater and marine.

B. Fish spoilage

Fishes are highly perishable and spoilage sets in soon after their landing. In the tropical climatic conditions, deterioration is rather rapid. The marketing of fish thus becomes a race against time to bring the fish to the consumers before the quality is reduced below an acceptable level. The pattern of fish spoilage is generally as follows: shortly after the death of the fish, glycogen in the muscles is converted into lactic acid and rigor mortis sets in, resulting in an increase in acidity and stiffening of the flesh. After rigor mortis, the flesh deteriorates and tends to become less stiff, limp and finally flabby. The spoilage is accompanied by an increase in pH of flesh which becomes somewhat alkaline in reaction; for a spoiled fish, pH may exceed 7.6 compared to 6.4 for an absolutely fresh fish (Anonymous 1962). Thus the stage of spoilage can be estimated by measuring the pH of flesh. (Khanna, 1970)

C. Fish Microflora:

The results of numerous studies indicate that fishes possess bacterial populations on in their skin, gills, digestive tract, and light-emitting organs. In addition, the internal organs (kidney, liver, and spleen) of healthy fish may contain bacteria, but there is debate about whether or not muscle is actually sterile (Brian, 2006).

The initial flora on fish is very diverse, although most often dominated by Gram-negative psychrotrophic bacteria. Fish caught in tropical areas, may carry a slightly higher load of Gram-positive organisms and enteric bacteria. Pathogenic bacteria that usually occur in fish include Enterobacteriaceae species, (*Salmonella, Shigella, Escherichia coli* etc.), *Pseudomonas*, *Staphylococcus aureus* etc. The Enterobacteriaceae include Gram-negative bacteria and all occurring on fish products as a result of contamination from animal\human reservoir. This contamination has normally been associated with fecal contamination or pollution of natural water or water environments, where organisms may survive for a long time or through direct contamination of products during processing. Besides bacterial contamination, fungal contamination is also quite prevalent in fish meat. (Huss, 1994).

D. Fish Preservation:

Fish should be subjected to proper preservation as soon as they have been caught to avoid spoilage. Modern preservation technique is lacking in Nepal so the unsold catch is either sun dried or smoked by using crude traditional method of fish preservation. These processed products due to rough packing are liable to undergo quick microbial infestation and thus have short shelf life. All these factors fail to fetch good market price. The methods of fish preservation such as freeze drying are still lacking in Nepal. The preservation pattern is also unhygienic. So contamination problem in preserved fish is acute.

Shrestha (1981) has reported in Nepal all fishes are edible. However, there has not been much study about the micro-flora contamination and chemical composition of these fish. Thus the present work here deals with the study of micro-flora and chemical composition of fish preserved by different methods.

Justification:

In spite of increasing demand of fishes, the fish markets of Kathmandu valley are not very well organized. They are generally congested, unhygienic and thus do not serve costumers in the way they have to be. The unhygienic conditions of fish markets make them susceptible to micro-floral contamination which, not only spoil the taste of the fish but also affect consumers adversely. It is thus very important to study the magnitude of contamination along with the study of nutritional values of fishes preserved in different ways.

Limitations:

Analysis of a few but important chemical constituents could not be undertaken due to limited resources and laboratory facilities. Microbial study of ice-preserved fish could also be of interest but because of greater volume of works for limited period of one year it was not carried out in this study.

CHAPTER II

OBJECTIVES

- Study about the hygienic condition of fish markets and different types of fish available in preserved conditions.

- Study of the different types of bacteria and fungi present in differently preserved fish available in the markets of Kathmandu.

- Analysis of the chemical compositions (protein and fat percentage) of fish preserved by different methods in order to find out their nutritional value.

CHAPTER III

LITERATURE REVIEW

Fish meat is considered as highly nutritious due to its high protein percentage. Besides being delicious, it is also easily digestible for people of all ages. Thus fish meat is considered to be good for health than the meat of other animal. People these days are very health conscious and thus the demand of fish in market is usually higher than that of other animal meat. However fish meat is a highly perishable commodity and the spoilage sets in soon after landing. Thus fish meat should be properly preserved to avoid spoilage. Preservation helps in maintaining a steady supply of fish in the market throughout the year including the period of poor catches and in keeping a control over the hike and fall in prices, thus stabilizing the price even in periods of low landings or bumper catches (Srivastava, 1985).

The hygienic condition of fish markets has not improved in last ten years. The separate fish market is still lacking. Chilled fishes are sold along the pavement making them susceptible to the microbial contamination. The quality of water used by the fish sellers to wash fish

The imperfectly or poorly preserved fish can result in serious food poisoning as they are contaminated by micro-flora. Besides, unhygienic condition of shops where these fish are sold can also bring about micro-floral contamination. Different species of bacteria and fungi invade preserved fish making them harmful for human consumption if not properly cooked.

Erichsen (1966), examined and classified bacterial cultures isolated from semi-preserved fish products. He found three groups of bacteria. One group belonged to the family Micrococcaceae, containing strains from the genera *Micrococcus* and *Staphylococcus*. The remaining two groups belonged to the family Lactobacteriaceae, comprising two species of the genus *Pediococcus* and hetero fermentative species of the genera *Lactobacillus* and *Leuconostoc*.

Kazanas (1966), made studies on the generic identification of bacteria isolated from nonirradiated and irradiated (0.3 and 0.6 Mrad) yellow perch fillets during the course of microbial spoilage have been conducted. After the enumeration and tabulation of

macro colonies on Petri dish cultures obtained from fillets, isolates were examined and keyed out essentially according to modified morphological and biochemical protocols of Shewan. Identification was further confirmed through reference to Bergey's Manual. Data obtained from each isolate were coded and recorded on IBM cards to facilitate identification. Total aerobic microbial plate counts obtained from nonirradiated perch before storage ranged from 105 to 106 microorganisms per gram of fish. Organisms isolated from these fillets, in order of decreasing number, consisted of *Achromobacter*, *Alcaligenes*, *Pseudomonas, Brevibacterium*, *Micrococcus, Flavobacterium*, *Bacillus*, *Sarcina*, *Microbacterium*, *Corynebacterium*, yeasts, *Lactobacillus*, *Vibrio*, *Aeromonas*, and a few Proteus and Escherichia cells. During storage and as spoilage progressed, the flora shifted and the pseudomonads became predominant. Irradiation of fillets to 0.3 and 0.6 Mrad reduced the aforementioned flora to the *Achromobacter-Alcaligenes* group, which constituted the residual flora throughout fillet storage.

Nauki (1975), examined Atlantic mackerel (*Scomber scomburs*) both its skin surface and the musculature prior to and after thawing, cleaning and washing and salting. Tested were also the cooking salt used, the brine, the conveyer bands, the vats for salting, the hands and clothing of the workers engaged as well as the water in the shop. Micro-flora such as coliforms, bacilli, moulds, yeasts, *Salmonellae* and pathogenic *Staphylococci* were isolated. It was found that during processing the microbial contamination of fish considerably rises.

Knochel and Huss (1984) showed that micro flora of salt herring regardless the fishing place consisted of 70% of moderate halophilic Gram-negative rods, 20% of Gram-positive cocci and 3% of yeasts.

Chaudhary (1989) isolated different types of microbial organisms in three different types of fish viz., *Labeo rohita, Harpadon nehereus, Puntius sophore* and *Schizothorax plagiostomus* selected as representatives of fresh, salted, sun-dried and smoked fishes respectively. His result showed the frequent occurrence of gram-ve bacteria in all types of fishes. The bacterial colonies which he isolated from *Schizothorax plagiostomus* were found to be *Pseudomonas*, *Leuconostoc*, *Micrococcus* and *Escherichia coli*. In case of salted fish (*Harpadon nehereus*) he isolated *Pseudomonas, Leuconostoc, Micrococcus* and *Lactobacillus* .The bacterial colonies which he isolated from *Puntius sophore* (sun-dried) were *Pseudomonas, Micrococcus, Leuconostoc* and *Escherichia coli*.

Koutsoumanis (2001), also worked on Gilt-head Sea bream (*Sparus aurata*), a Mediterranean fish of high consumption and commercial interest in Greece was studied. Eleven replicated storage experiments were carried out with ungutted fresh gilt-head sea bream. Fish were bought from the Nireus Aquaculture Industry in Athens within 6 to 12 h after catch and then transported in ice, within 30 minutes, to the laboratory. The fish were then stored under controlled isothermal conditions (from 0 to 15°C) in high-precision low-temperature incubators. The samples were taken at appropriate time intervals to allow for an efficient kinetic analysis of sensory quality and microbial growth. Pseudomonads were established as the main bacteria of Mediterranean gilt-head sea bream (*Sparus aurata*) stored under aerobic conditions at a temperature range from 0 to 15°C.

Dąbrowski *et al*. (2001), research covered qualitative and quantitative analyses of micro flora of traditionally and vacuum-packed low-salt herring fillets. Smaller amount of psychrophiles and yeasts was found in traditionally packed samples than in vacuum-packed proving the difference between tested products. According to their results Gram-positive cocci were represented by *Micrococcus* spp. in traditionally and vacuum-packed products. Traces of *Staphylococcus* spp. were additionally isolated from traditional packaging. Gram-positive rods: *Corynobacterium* spp*.* and *Celullomonas* spp*.* were found in traditionally packed herring whereas *Brevibacterium* spp. was detected in vacuum-packed samples. Gram-negative rods were represented by *Pseudomonas* spp*.* and *Xanthomonas* spp. They were found in traditionally packed samples as well as in vacuum-packed. In the latter *Escherichia* spp*.* was also found.

Tryfinopoulou *et al*. (2002), worked together on gilt-head sea bream Mediterranean fish (*Sparus aurata*) stored under different conditions. They isolated different strains of *Pseudomonas* from every sample.

Gonzalez *et al.* (2002), worked on 54 packages (each one belonging to a different lot) of vacuum-packed cold-smoked salmon (30) and trout (24) produced by six Spanish smokehouses. Sensorial, chemical, physicochemical and microbiological characteristics were examined. They isolated Enterobacteriaceae, lactic acid bacteria (LAB), fungi and presumptive aeromonads and staphylococci from these fishes. *Listeriae* other than *L. monocytogenes* were isolated from three packages. Among 377 bacteria randomly isolated from plate counts, LAB predominated, with *Carnobacterium* (*C. piscicola*) and *Lactobacillus* (eight species) being the genera most frequently found. The second and third major groups were Enterobacteriaceae and Micrococcaceae, respectively. *Proteus vulgaris*, *P. mirabilis* and *Serratia liquefaciens* were dominant among Enterobacteriaceae and coagulase-negative staphylococci among Micrococcaceae. Minor microbial groups such as aerobic gram-negative bacilli (*Acinetobacter; Moraxella and Pseudomonas*), *Brochothrix, Aeromonas, Bacillus* and *Vibrio* were also found.

Frazier and Westhoff (1978) found that molds are the chief spoilage organisms on smoked fishes. The incipient growth of molds makes the surface of the fish sticky to touch. It gives a musty flavour to fish in the vicinity of their growth.

Lowery (1984) found that yeast and mould are usually play merely a minor role in the spoilage of fish because they constitute only a small portion of the initial micro flora and because they grow slowly compared with most bacteria. The small growing molds can spoilage fish by forming visible colonies but this occurs only when storage conditions selectively inhibit bacterial development. He also pointed out that mold spoilage occurs when the environmental conditions such as temperature, water activity and pH are unfavourable for bacteria.

Atapattu and Samarajeewa (1990) studied the microflora of dried-salted fish from markets in Kandy, Sri Lanka with emphasis on visibly spoiled dried fish. A total of 61 fungal isolates from 25 dried-fish were isolated and identified. The most prevalent fungus was *Aspergillus niger*. Species of *Aspergillus flavus, A. fumigatus, A. glaucus, A. restrictus, Aureobasidium* spp. *Basipetospora halophila* (a genuinely halophilic fungus) *Cladosporium herbarum, Gliomastix* spp., *Penicillium chalybeum* and *Penicillium expansum* were present.

Fafioye *et al*. (2002) worked together on the samples of traditionally smoke-dried *Clarias gariepinus* (Burchell), *Chrysichthys nigrodigitatus* (Lacepede), *Sarotherodon galilaeus* (Trewavas), *Heterotis niloticus* (Cuvier) and *Heterobranchus bidorsalis*

(Geoffroy) obtained from Oja Oba Market, Ago-Iwoye, Nigeria and examined for fungal infestation. The fish samples incubated on Potato Dextrose Agar (PDA) for 7 days showed fungal infestation. Fungi isolated and identified included *Mucor* spp., *Aspergillus* spp., *Rhizopus* spp. and *Fusarium* spp. Six fungal species were isolated from *C. nigrodigitatus*, five each on *C. gariepinus* and *H. niloticus*.

Chaudhary (1989) isolated the number of yeast and mold colonies from chilled and dried fishes. In chilled fish (*Labeo rohita*), he isolated about 150 and 40 yeast and mold colonies respectively. In sun-dried fish (*Puntius sophore*), the number of yeast and mold colonies which he isolated were 90 and 70 respectively, in 1ml. sample of the fish. Similarly, he isolated 260 and 270 colonies of yeast and mold respectively from 1ml sample of smoked fish (*Schizothorax plagiostomus*).

According to Chaudhary (1989), the protein content of smoked fish (*Schizothorax plagiostomus*) was about 60.31 percent, that of salted fish (*Harpadon nehereus*) was 59.03 percent and the protein content of sun-dried fish (*Puntius sophore*) was 59.67 percent. Similary, the fat content of smoked fish was about 7.86 percent, that of salted fish was about 8.33 percent and the fat content of sun-dried fish was 8.00 percent.

Several scientists have worked on the chemical composition of fishes. Foda *et al*. (1986) studied the chemical constituents of Bolte fish and reported that small fishes contain higher percentage of waste protein in relation to larger one.

Leekang (1987) studied the lipid composition and seasonal variation in fatty acid composition of body oil in several fishes.

CHAPTER IV

MATERIALS AND METHODS

A. Study Area:

The study areas for present work are two districts of Bagmati zone, Kathmandu and Lalitpur. For the study of chilled fish, Kalimati and Kichapokhari of Kathmandu, and Lagankhel of Lalitpur were selected. Similarly, for the study of smoked, salted and sun-dried fishes, Mangal Bazaar of Lalitpur and Asan of Kathmandu were selected.

B. Market Survey:

The study was conducted by direct observation of fish markets and shops and by interviewing the shopkeepers. The survey period lasted for about a month during Oct\Nov. 2006. During survey, the fish markets of Kathmandu and Lalitpur were visited frequently. Prior to such visits, questionnaires were developed to interview shopkeepers in order to gather enough information about different types of preserved fishes; mostly consumed ones; the best season for selling fishes; price of different fishes etc. A model of questionnaire is included in appendix-I.

The hygienic conditions of different fish shops were also taken into consideration. Questions were asked to the shopkeepers regarding the improvement of hygienic condition of shops. Even though some shopkeepers hesitated to give honest answers, however, the overall response from the shopkeepers was good enough to gather the required information. Several photographs of the study area, shops and different types of fish displayed for selling were also taken.

C. Sample Collection:

For the study of micro flora present in the preserved fish available in the Kathmandu market, three different types of fish samples were selected. The numbers of samples were total of five for each type of fish. The collection was done in the local market of Lalitpur. These included traditionally smoked *Cirrhinus mrigala*; sun dried *Oxygaster* spp. and salted *Harpadon nehereus.* Sterile plastic bags were used to store the collected samples.

After completing market survey and sample collection, microbial analysis of selected fish samples was performed. For the analysis micro-flora viz; bacteria and fungi present in the collected fish samples were brought into the science laboratory of NAST, Khumaltar, Lalitpur. The lab work at NAST lasted for about six months starting from late April 2006 to early September 2006.

Microbial analysis was followed by the analysis of chemical constituents of the selected fish samples. For this purpose the samples were taken to The Central Food Research Laboratory, Babar Mahal. Here the laboratory work lasted for about two weeks during late September 2006.

D. Microbial Analysis:

For microbial analysis, one smoked fish, one salted fish and two sun-dried fish and about 1gm from the head and the body parts of each type of fish samples were taken.

For easy growth of micro flora two types of agar media were selected; Nutrient Agar (NA) for the growth of bacteria and Potato Dextrose Agar (PDA) to grow fungi.

Agar is a dried polysaccharide extract obtained from species of red algae, and used as solidifying agent of various types of microbiological media. Nutrient agar is a commonly used food medium for bacterial cultures. It contains:

- beef extract (provides carbohydrates, nitrogen, vitamins, salts)
- peptone (helps control pH)
- agar (a carbohydrate used as a solidifying agent)
- distilled water (an agent for distributing food materials to growing colonies of micro-organisms).

I. Bacterial Culture

The collected fish samples were first subjected to bacterial culture which involved following steps:

i. Media preparation:

It is the first step in any microbial analysis. It involved following steps:

- The glass wares required for the microbial analysis such as Petri plates, pipettes, glass rods etc. were first sterilized at 170^{o}C in oven for at least an hour.
- At least 10 Petri discs were first cleaned and sterilized before pouring the media into them.
- For 10 plates, 200ml of media was required. Thus required amount of Nutrient Agar was weighed and taken in clean conical flask and 200ml of distilled water was poured into it.
- The mixture was stirred thoroughly and heated for a few minutes so that the agar would dissolve completely.
- The mouth of the flask was sealed air tight with cotton plug and then wrapped with aluminum foil and sterilized for 45 minutes at 100^{o}C in an autoclave.

ii. Serial Dilution:

It is important to know how many live bacteria are present in the each fish sample. Performing such determinations demonstrates that if bacteria are alive or viable, they generally capable of forming colonies in or in a solid medium. Therefore the number of cells in specific volume of material capable of dividing on or in a solid agar medium is referred to as viable number. For practical reasons, however the viable number is expressed in terms of colony forming units (CFUs).

Determining viable number in a culture requires a procedure to dilute or sufficiently separate bacteria so that they can form separate colonies. Because such cultures and specimens usually contain several thousands or even hundreds of millions of cells per milliliter, dilutions must be performed before plating of culture samples takes place. A **serial dilution procedure** is a method of sequentially diluting a culture sample through a series of sterile dilutions blanks. The fluid in the dilution blanks is usually distilled water.

During dilution, by transferring 1.0ml (milliliter) from the original culture to 9ml of dilution blank (diluent), a 1:10 (one-to-ten) dilution is obtained. The dilution also can

be expressed as 10^{-1} or 1\10. Taking 1.0mL from this 1:10 dilution and placing it into another 9mL of dilution blank results in a 1:100 or 10^{-2} dilution. The dilution in this test tube is calculated by multiplying the two *dilution factors*, $10^{-1}X10^{-2}$ or 1:100. The dilution (10^{-2}) is referred to as the *dilution factor*. The delivery or transfer of culture or fluid volumes is done by means of pipettes.

a. Materials required for Serial Dilution:

- Twenty-four 9mL sterile water dilution blanks in cotton plugged test tubes
- Eighteen individually wrapped 1mL sterile pipette
- Pi-pump
- Test-tube stands
- Vertex (shaker)

b. Procedure in Serial Dilution:

Prior to serial dilution, the fishes were cleaned and the heads were separated from their respective bodies. The body and the head parts of each fishes were crushed separately into fine powder using a sterilized mortar. Serial dilution of head and body parts were performed separately. Following steps were involved during sterile dilution of head and body parts of each type of fish samples:

- To make distilled water dilution blanks, 24 test tubes were taken and 9 ml of distilled water was poured into each tube and autoclaved.
- Out of 24 sets of dilution blanks, nine sets were labeled as 10^{-1}, 10^{-2} and 10^{-3} to make the dilutions of head region of each fish sample.
- Nine other sets were also labeled as 10^{-1}, 10^{-2} and 10^{-3} to make the dilution of body region of each fish sample respectively.
- One gm of crushed body of sun-dried fish was weighed and then diluted in one of the remaining 9ml of dilution blanks. This was considered as the original solution and the contents were mixed well by placing the test tube in the vertex.
- One mL sterile pipette was carefully unwrapped and attached to Pi-pump.
- With the help of pipette and Pi-pump, 1 mL of original solution was transferred to the first 9mL dilution blank (10^{-1}).
- The pipette was again carefully disconnected and placed in container.
- Second sterile 1mL pipette was connected to the Pi-pump to transfer aseptically

1mL of the 10^{-1} to the second 9mL dilution blank 10^{-2}.

- The pipette was carefully disconnected and placed in container.
- Third sterile 1mL pipette was connected to the Pi-pump to transfer aseptically 1mL of the10^{-2} to the third 9mL dilution blank 10^{-3} and mixed well using vertex.
- The pipette was disconnected and kept in a container.
- The contents of the dilution blanks were mixed well using vertex.
- The respective dilutions were saved for next procedure.

In the same way, the sterile dilution of the head of sun dried fish and sterile dilutions of head and body parts of rest of the two fish samples were performed.

iii. Pour Plate and Streak Plate:

The viable numbers of colony-forming units (CFU) of bacterial suspension can be determined by use of the *pour plate technique* or *spread plate technique*. Pour plate method involves the pouring of warm media with temperature of about 45^{o}C over the 1mLof dilution.

a. Materials required:

- Eighteen Petri plates
- Eighteen dilution blanks saved from earlier procedure
- Individually wrapped 18 sterile pipettes
- Pi-pump

b. Procedure involved:

- The media was first autoclaved and then cooled to about 45^{o}C.
- For each fish samples, six sets for Petri plates were separated for head and body regions respectively.
- Out of six Petri plates in all cases, three were separated and labeled as 10^{-1}, 10^{-2} and 10^{-3} and three were labeled as 10^{-1}, 10^{-2} and 10^{-3} respectively in order to pour the respective dilution blanks.
- Each plate was also tagged with the name of fish samples.
- To each labeled plates, one mL from each 18 dilution blanks was added with the help of sterile pipettes attached to Pi-pump.
- The media was cooled to about 45^{o}C and was poured into each plates and left to set.

- The plates were sealed with Paraflim tape and stored in the incubator in an inverted position for the culture of bacteria.
- The plates incubated for two days at 34^{o}C and the bacterial colonies were counted.

iv. Colony Count:

Pour plate is followed by colony count. If the colonies are large enough to count easily with unaided eye plates can be held.

- The position of each colony on the bottom of the plate was marked with a marking pen as counting it.
- Plates containing more than a few hundred colonies were considered "too numerous to count" (recorded as TNTC in data records).
- The ratio was calculated for each dilution step by dividing the number of colonies on each plate by the number on the next more dilute plate.

It can be expressed as follows.

$$\frac{\text{(CFUs) Colony forming units}}{\text{mL (Original sample)}} = \frac{\text{Average number of colonies}}{\text{Plate}} \times \text{Dilution factor}$$

Here,
Dilution factor refers to the total number of times the original sample was diluted.

v. Sub-culture:

For the identification of each individual colony, it was necessary to transfer them in separate NA plates so that pure cultures can be obtained. Thus sub-culture involves the transfer of each individual colony from mixed culture plates to sterile NA plates to obtain pure culture. It involved following steps:

- Petri plates having an average of 30 to 50 colonies were selected for sub-culture.
- With the help of an inoculating loop, a little amount of culture from an isolated colony belonging to the mixed culture plate was removed with the help of a well sterilized loop and streaked in a new agar plate.
- The bottom of the Petri plate was labeled with the fish sample and dilution times and was incubated for 24 to 48 hours at 34^{o}C in inverted position.

II. Identification of Isolated Bacteria:

i. Gram Stain:

Gram staining of bacteria growing on each plate was done and the slides were observed under microscope. Gram stain technique is used to divide bacteria into two major groups. The Gram differentiation is based upon the colour reactions exhibited by bacteria when they were treated with the crystal violet dye followed by iodine-potassium solution. Certain organisms lose the violet colour rapidly when ethyl alcohol is applied, while others lose their color more slowly. After the decolourization step, a counter stain-usually saffarin is used. Bacteria resistant to decolourization will retain a blue or purple colour and will not take counter stain. Such organisms are referred to as **gram positive**. Those microorganisms unable to retain the crystal violet stain will take the counter stain and consequently exhibit a pink or red colour. The term **gram negative** is used to describe these organisms (Wistreich, 1997).

a. Differentiation of Gram-positive and gram-negative cell wall:

Treatment of cells first with the crystal violet and then with Gram's iodine results in the formation of large, insoluble dark purple, almost black, dye-iodine complexes inside cells. Such dye-iodine complexes are retained in gram-positive by the thick peptidoglycan meshwork in their cell walls. In gram-negative cells the crystal violet-iodine complexes are readily released through the very thin peptidoglayer remaining after their membranes have been dissolved during decolourization step.

Several factors can affect the Gram reaction, including cell age, autolysin levels and growth conditions. As culture becomes old, bacterial walls become more permeable to the crystal violet-iodine complex and therefore tend to exhibit gram-negativity more readily. Situations of this type would have significant effects on gram-positives because cell walls would become leaky and allow the cells to be easily decolourized.

b. Procedures involved in Gram-staining:

- A thin film of the material to be examined was prepared and dried.
- The material on the slide was heat fixed and allowed to cool before

staining.

- The slide was flooded with crystal violet stain and allowed to remain without drying for 10-30 seconds.
- The slide was rinsed with tap water, shaking of excess.
- The slide was flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface.
- The slide was rinsed with tap water, shaking off excess.
- The slide was flooded with alcohol acetone decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer. Thicker smear requires more aggressive decolorizing.
- The slide was flooded with counterstain (safranine) for 30 seconds and washed off with tap water.
- The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100X objective.

Gram-Stain procedure can be interpreted as in table-1:

Table-1: Gram Satin Procedure

Reagent	Cells Colour Results	
	Gram Positive Cells	**Gram negative Cells**
Crystal violet (primary stain)	purple	purple
Gram's iodine (mordant)	purple	purple
Acetone alcohol (decolourizer)	purple	no colour
Safranin (counterstain)	purple	red

ii. Biochemical Tests:

The biochemical test was performed to identify the unknown organisms. Bacteria are among the most diverse organisms with respect to the types of enzymes they contain. Thus biochemical test is necessary for the identification of unknown bacteria species. A variety of specialized media are designed to

facilitate determining the biochemical reactions of bacteria. These include: Methyl Red and Voges-Proskauer (MR-VP), Indole, Urease, Oxidative\Fermentation (O\F), TSI (Triple Sugar Iodine) and finally the Citrate Utilization Tests.

5ml of each of these media were prepared with distilled water and taken in the required number of test tubes and sterilized in autoclave at 45^{o}C. Methyl Red and Voges-Proskauer (MR-VP), Urease and Oxidative\Fermentative (O\F) were in form of broth while SIM (Sulphide-Indole-Motility Medium) and Indole formed solid media. SIM media is used for the detection of H_2S, Indole and Motility. TSI and Citrate were also in form of solid but it was necessary to form slants of these media. Once the media were cooled and slants have been properly made, they were inoculated with unknown bacteria species and incubated overnight at 34^{o}C. The next day, each test tube was properly observed and the results were noted down.

a. Catalase Test:

Catalase is an enzyme that splits hydrogen peroxide into water and oxygen. Hydrogen peroxide is produced as a byproduct of respiration and is lethal if it accumulates in the cell. All respiring organisms therefore must have some mechanism for detoxification. Catalase is one of the common methods. In this method, hydrogen peroxide is added to a colony with the help of a glass rod. In case of catalase-producing bacteria, it is broken down and the oxygen that is produced can be seen as bubbles.

b. Oxidase Test:

Oxidases are the enzymes that catalyze the reduction of oxygen during respiration. For example, in most gram positive bacteria and many gram negative bacteria cytochrome oxidase performs the final step in electron transport, reducing oxygen to water. Other bacteria, such as the Enterobacteriaceae, do not reduce oxygen using this enzyme. Thus detection of cytochrome oxidase is a valuable tool in differentiating among bacteria. The test utilizes a colorless reagent to detect oxidase. This chemical in the presence of oxygen and an oxidase enzyme will form a colored compound.

In this process, a sterile swab was used to transfer a heavy inoculum of the bacteria to a slide. The slide was placed on a white paper and the oxidase

reagent is added to it. A change in colour is then observed. A positive reaction appears pink, then maroon and finally black.

c. Methyl Red and Voges-Proskauer Tests (MR-VP):

These fermentation tests are used to differentiate between certain intestinal bacteria called coliforms. The MR-VP broth contains dextrose as the carbohydrate source. Some coliforms will ferment the dextrose to acid products that will cause the pH to drop below pH 5. This is called a mixed acid fermentation. After incubation the addition of methyl red, a dye which turns red below pH 4.4, will indicate whether such fermentation has occurred. Other coliforms will convert dextrose to less acidic products such as ethanol or butanediol. These bacteria are negative in the methyl red test.

Butanediol fermentation is demonstrated by the Voges-Proskauer test which measures the presence of acetoin (acetyl methyl carbinol), a precursor to butanediol. This test uses the same medium as the methyl red test and both tests are usually performed in parallel. Barritt's reagents, alpha-naphthol and potassium hydroxide, are added in the ratio 3:1 to a 48 hour culture and the tube is shaken to aerate the solution. The development of a pink or red color after agitation is a positive reaction for the production of acetoin.

d. Indole Test: The ability to degrade amino acids to identifiable end products is often used to differentiate among bacteria. Tryptophan, for example, is hydrolyzed to indole, pyruvic acid and ammonia by tryptophanase. The pyruvic acid can be further metabolized to produce large amounts of energy. The ammonia is available for use in synthesis of new amino acids.

Indole is detected by reaction with Kovac's reagent (paradimethylaminobenzal-dehyde in alcohol) to produce a red color.

e. OF Test (Oxidative\Fermentative):

Oxidative-fermentative tests are used to differentiate organisms that oxidize carbohydrates from those organisms that ferment carbohydrates such as members of Enterobacteriaceae. Hugh-Leifson Medium is used for this test.

f. Urease Test:

Urease is an enzyme that breaks the carbon-nitrogen bond of amides to form carbon dioxide, ammonia, and water. Members of genus *Proteus* are known to produce urease. Urease can be detected by plating bacteria onto an amide containing medium, specifically urea. When urea is broken down, ammonia is released and the pH of the medium increases (becomes more basic). This pH change is detected by a pH indicator that turns pink in a basic environment. Phenol red indicates a positive test for urease.

g. Citrate Utilization Test:

For this test, Simmons Citrate agar is used as a medium. It contains citrate as the sole carbon source and ammonium salts as the sole nitrogen source. Organisms that metabolize citrate utilize the ammonium salts releasing ammonia and increasing the pH of the medium. Bromothymol blue is present in the medium as the indicator dye. It is green at neutral pH and deep blue above pH 7.6.

h. SIM (Sulphide-Indole Motility) Test:

SIM derives its name from three reactions: production of hydrogen sulfide from sulfur-containing amino acids, *indole* production and motility. The motility is checked by observing migration of the inoculums from the stab line through the semisolid medium. The liberation of hydrogen sulfide is indicated by a blackening of the medium. Indole production is determined after the addition of Kavoc's reagent. The medium is used primarily for differentiation of gram negative enteric bacteria.

i. Triple Sugar Iron (TSI) Test:

TSI is widely used in the identification of gram negative bacteria particularly the Enterobacteriaceae. TSI contains sucrose and lactose in addition to the dextrose. The media is poured as slants and are inoculated with a stab to the butt followed by a streak of the slant surface. The bacteria therefore are exposed to both an anaerobic environment (butt) and an aerobic one (slant). Phenol red is present as an indicator. Do not tighten the cap on the tube.

If the bacteria are non-fermenters, such as *Pseudomonas*, they can grow on

the slant by the aerobic degradation of protein components in the medium to alkaline products. The slant and the butt will remain red.

If the bacteria can ferment dextrose, but not sucrose or lactose, acid is produced in the slant and the butt and the medium turns yellow. The dextrose is used up, however, within 12 hours. Bacteria at the surface continue to grow by degrading proteins. By 18 to 24 hours, the alkaline end products cause the medium in the slant to revert to a red color. Such reactions are characteristic of *Shigella a*nd other nonlactose fermenters.

If the bacteria can ferment lactose and/or sucrose as well as dextrose, the slant and butt will remain yellow after prolonged incubation. The high concentration of lactose keeps the slant acidic despite the production of alkaline products by protein degradation.

TSI also contain sodium thiosulfate and ferrous sulfate as indicators of hydrogen sulfide production. *Salmonella* spp. (dextrose fermenters, lactose nonfermenters) will yield an acid butt with a black precipitate and an alkaline slant. TSI reaction can be interpreted in the following ways depending upon the organism being tested:

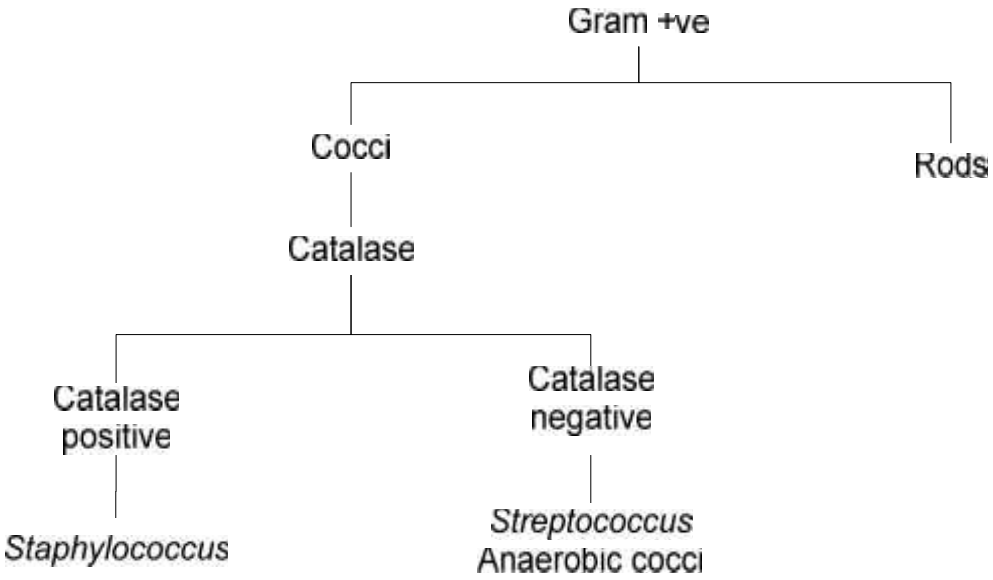
Table-2: TSI reaction*

S.No.	Reaction (Slant\Butt)	Sugar Attacked
1.	Alkaline\Acid Red\Yellow	Only glucose attacked
2.	Acid\Acid Yellow\Yellow	Glucose and lactose attacked or, glucose and sucrose attacked or, glucose, lactose and sucrose attacked.
3.	Alkaline\Alkaline Red\Red	Neither glucose nor lactose attacked, peptones utilized
4.	Alkaline\No change No colour change in slant and butt	Neither glucose nor lactose attacked, peptones utilized

***Source of above table: Bhattrai (2001).**

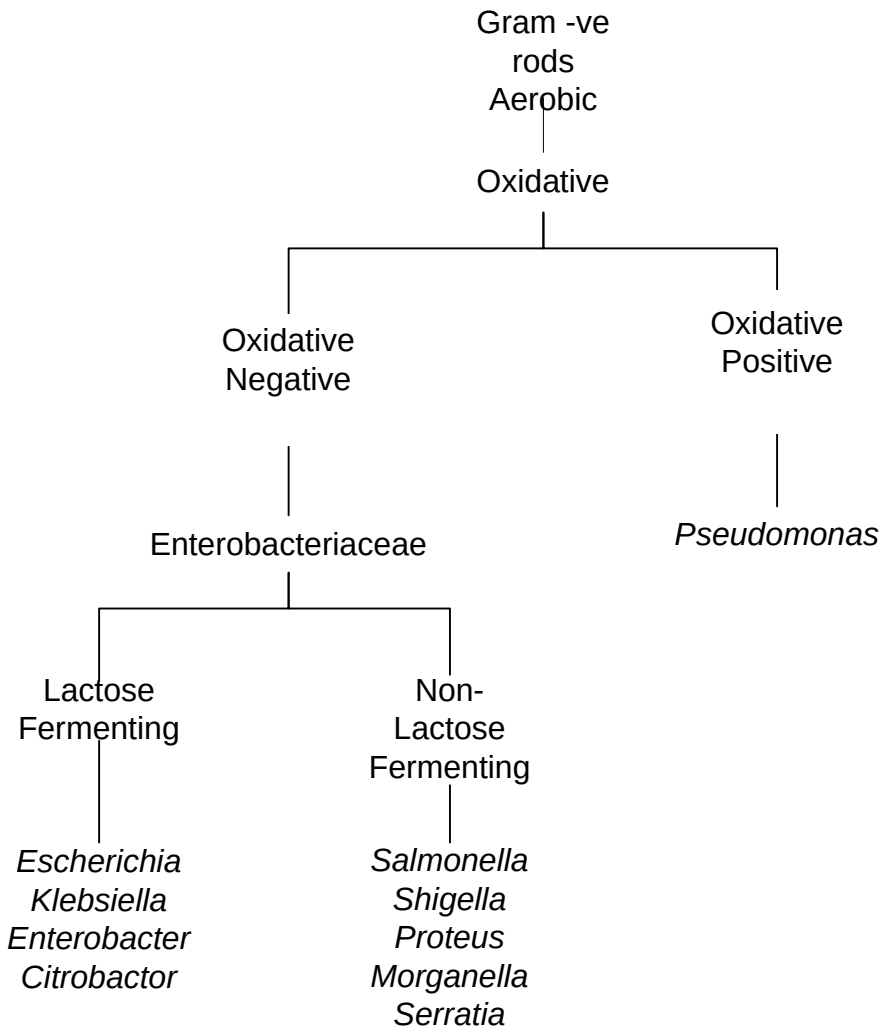
With the end of biochemical test, the unknown gram-negative bacteria species were identified. Identification of bacteria were done according to Cheesbrough (1984).

Fig. 1 Identification Chart for Gram Positive Bacteria



Source of above chart: Cheesbrough (1984).

Fig. 2 Identification Chart for Gram Negative Bacteria



Source of above chart: Cheesbrough (1984).

III. Fungal Culture

After the culture and identification of bacteria, the fish samples were subjected to

fungal analysis. Steps involved in the culture and identification of fungi are as follows:

i. Media preparation:

About 39.3gm of Potato Dextrose Agar (PDA) was taken in a conical flask and 14mL of distilled water was added to it. The media was heated for a while so that it dissolved well. The conical flask was then sealed with cotton plug and aluminum foil and was sterilized in autoclave.

ii. Serial Dilution:

The serial dilution of the head and the body parts of each fish sample was performed in the same way as in case of bacterial culture. In this case also 24 dilution blanks were made. For each fish sample, six sets of dilution blanks were made for head and body regions respectively. Thus the total of eighteen such dilution blanks were made and saved for further procedure.

iii. Pour Plate:

About one mL from each eighteen dilution blanks was poured into the respective sterilized Petri plates with the help of eighteen individually wrapped sterilized pipettes. The sterilized media was allowed to cool to about at least 45^0C and then poured into each plate. The plates were then incubated at 28^0C for five days.

iv. Colony Count:

On the fifth day of incubation, each Petri plates were carefully taken out of the incubator and the fungal colonies were observed and counting was done. The counting was done in the same way as that of bacteria.

v. Sub-culture:

For the growth and identification of each individual colony, sub-culture was necessary. Thus a loopful from each of these colonies was taken with the help of sterilized loop and subculture into another PDA plates so that pure cultures were obtained. Once the pure culture was obtained, slides were prepared for identification.

vi. Preparation of Slides:

Once pure culture was obtained, slides of those cultures were prepared for the identification of cultured species.

a. Materials required:

- Clean glass slides
- Teasing needles
- Lactophenal cotton blue solution
- Cover slips

b. Procedures involved:

- For slide preparation, a portion of the fungal culture was removed with the help of dissecting needles.
- Wet mount was prepared using lactophenal cotton blue solution as the suspending material.
- The fungal growths were teased apart with the aid of dissecting needles before applying the cover slip.
- Each slide was then observed under microscope for identifications. The photographs of culture plates were also taken frequently.

In this way microbial analysis of each fish samples was performed in the laboratory of NAST.

Source of above mentioned methods of microbial analysis- Wistreich (1997).

E. Chemical Analysis:

The chemical analysis of each fish samples were performed at The Central Food Research Laboratory, Babar Mahal. For the determination of chemical composition, the fish samples choose were the ones used for microbial study. These included about 200gm of *Naini* (smoked), 200gm of *Harpadon nehereus* (salted) and 200gm of *Oxygaster* spp. (sundried) respectively. These fishes were first dried at 800^0C and grind into fine powder and stored separately in air tight apparatus as main sample stock. This sample stock was used for the determination of protein and fat.

I. Determination of Protein:

There are number of methods for the quantitative estimation of protein. Here crude protein was determined by estimating the total nitrogen by Kjeldahl method. Kjeldahl method is simple but accurate method to estimate total protein. Total nitrogen, as estimated by the Kjeldahl method, multiplied by a factor 6.25 gives the value of total protein. It can be expressed as following:

Total protein= total nitrogen N_2 x 6.25 (Bajrachrya 1999).

i. Materials required:

- Conc. H_2SO_4
- NaOH
- Distilled water
- Boric acid
- Mixed indicator solution
- Kjeldahl-flask
- Bunsen burner
- Conical flask
- Burettes
- Pipettes

ii. Procedure involved:

- From the main sample stock of smoked fish, about one gram of the sample was kept in Kjeldahl flask and two grams digestion mixture and 5ml. of concentrated Sulphuric acid were added.
- This flask was kept in Kjeldahl digestion system and boiled for 30 to 40 minutes. After 40 minutes the mixture turned into crystal form and settled in the bottom of flask.
- A small quantity of distilled water was added in the flask and shook well till it dissolved completely. The solution was transferred to a measuring flask of 150ml. and distilled water was filled up to the mark.
- 10ml of Sodium hydroxide was added to 10ml. of the sample solution and was kept in the Nitrogen distillation system.
- 10ml of Boric-acid kept in a measuring flask of 100ml. was kept under this system to absorb the discharged ammonia from the

solution kept in Nitrogen distillation system.

- When the colour of Boric acid in measuring flask became dark green and it was titrated against Sulphuric acid until the dark green colour turned into light red.

- The consuming volume of Sulphuric acid in the burette was noted to calculate the protein percentage.

In this way the process was repeated again following the same procedure for another 1gm of sample in order to compare the data. The crude protein content of the other two fish samples were also determined in same way as mentioned above.

II. Determination of Fat:

For the extraction of fat, Soxlet Extractor Method was used. In this method fat is extracted by distillation with organic solvent (Bajracharya 1999).

a. Materials required:

- Diethyl ether (or petroleum ether 60-80^{o}C)
- Soxhlet apparatus
- Round bottom flask

b. Procedure involved:

- A round bottom flask was taken and it weight was measured.
- From the main powder sample stock of smoked fish, about three grams was taken and kept in thimble.
- The sample was extracted with the help of petroleum ether for eight hours on Soxlet extractor.
- Then the round bottom flask was taken out and kept in the oven for about an hour. The flask was kept in the desiccators to cool.
- The weight of round bottom flask with remaining sample was taken.

The process was repeated all over again following the same procedure for the sample in order to compare the data. Similarly, the powder of other two fishes was also subjected to the same experiment as mentioned above to determine

the fat content present in them.

$$\text{Fat percentage on wet basis} = \frac{\text{weight of fat}}{\text{Weight of sample}} \times 100$$

Where,

Weight of fat = weight of round bottom flask + remaining sample- weight of round bottom flask.

CHAPTER V

OBSERVATIONS AND RESULTS

A. Fish market survey:

The number of fish markets in Kathmandu has increased considerably in the last few decades. There are many fish markets in Kathmandu as well as in Lalitpur. Some popular fish markets are at Ranamukteshowar, Asan, Kalimati, Indrachoke, Mangal Bazar, Lagankhel etc. The fish market of Kalimati is quite large in comparison to the other the fish markets and they exclusively sell fishes in chilled condition. There are about ten fish shops where fishes brought from various parts of Nepal and also those from India are sold. These fishes are preserved in ice boxes in order to prevent them from spoilage. In older days there were no such separate fish markets in Kathmandu valley but now due to high demand of fish separate markets are being opened.

Ranamukteshowar of Kathmandu is one of the famous and busiest spots for buying chilled fishes. Here different species of fishes brought from Terai and India are sold. One of the popular spots for buying chilled fishes in Lalitpur is Lagankhel. Many fish-sellers are found along the streets selling fishes in baskets or displaying them on small tables especially in evenings. Some fishes such as Mungri (*Clarias batracus*) are sold alive in large tin vessels filled with water. People returning home from their works stop by to buy the fishes of their choice.

Apart from fishes, some fresh aquatic crustaceans are also available in a few fish shops of Kathmandu, mainly at Ranamukteshore. These include crabs, lobsters, shrimps, prawns etc. imported from India. Prawns are best preferred among these, and are sold in high prices. Big hotels and restaurants have high demand for prawns and are served as special delicacy. In spite of being delicious, demand of these crustaceans is rather low in comparison to the fishes. It can be mainly because of two reasons; firstly because local people are not aware about the nutritive value of crustaceans and secondly, the price of crustaceans, like prawn, shrimps, lobsters are

pretty high to be afforded by local people.

I. Hygienic condition of fish market:

In spite of the increasing demand of fishes, the marketing of fish in Kathmandu valley is not very well organized. The fish markets are generally congested and unhygienic and thus do not attract sufficient costumers. There has been no remarkable improvement of fish markets in last ten years. The physical facilities like quality of tap water, cold storage, preservatives and sanitary condition are still unsatisfying. The reason behind such the poor condition of fish markets is the lack of proper management. Besides, Government has also not paid enough attention towards this field.

In the fish market of Ranamukteshore, fishes are sold in small shops along the road by displaying them on tables. Besides fish meat, buff, mutton, pork, chicken, sausages are also sold together. This makes fishes quite susceptible to all kinds of infestation. In the fish market of Lagankhel also, fishes are sold along the pavement where they can easily get contaminated by dust and dirt. Flies are also seen hovering around them to bring about more contamination. The quality of water used to keep these fishes in fresh condition is also not quite satisfying. The shopkeepers were seen using same water for many days due to inadequate supply of water. Besides, there is no proper place in any of the fish markets to dispose wastes such as scales, gills and viscera of fishes. Shopkeepers simply thrown them away without any precautions, unaware about the infestations they can bring.

Smoked, salted and sun-dried fishes are sold in open containers making them susceptible to microbial contamination. Various dried food stuffs such as beans, dried potato slices, crackers etc. are sold along with these fishes.

II. Preserved fishes available in the markets of Kathmandu:

Fishes preserved by four different methods are available in the markets of Kathmandu. These include:

a. Chilled (Ice-preserved)

b. Smoked fish

c. Sun-dried fish

d. Salted fish

Among these four types, fishes are mostly available in chilled condition in the fish markets of Kathmandu.

a. Chilled fishes:

Both exotic as well as indigenous fishes are sold in chilled form. These fishes are kept in ice boxes or refrigerators in order to prevent them from spoilage. Exotic fishes include both marine as well as fresh water fishes such as Tuna, Pomfert, *Cyprinus carpio*, *Hypopthalmichthys molitrix*, *Ctenopharyngodon idella, Aristichthys nobilis* etc. These fishes are imported mainly from Andra Pradesh, Bombay, Calcutta of India and some are also imported from Bangladesh. The indigenous species include Rohu (*Labeo* spp.), *Catla catla*, Buhari *(Wallago attu),* and *Channa* spp. Mungri (*Clarius batracus*), JalKapoor (*Clupisoma gaura*), Naini (*Crihhinus mrigal),* Sahar (*Tor* spp.) etc. These fishes are generally brought into the valley from different parts of Nepal such as Janakpur, Rajbiraj, Birgunj, Trishuli etc.

According to the survey done in the different fish markets of Kathmandu, the most preferred among these fishes is Rohu (*Labeo* spp.) due to its delicious meat. Similarly, the most expensive fresh fish is Katle. Among exotic species, the most expensive ones are Tuna and Pomfert which are imported from the seas of India. Pomferts are one of the highly priced marine fishes and thus its demand in local market is quite low. Besides, it is not available in every fish shop.

b. Smoked fishes:

Like chilled fishes, smoked fishes are also quite popular among the people of Kathmandu. In Lalitpur, smoked fishes are available in several shops at Mangal Bazaar. Asan is one of the most popular places for buying these types of fishes. There are many small and big shops here where varieties of such fishes are available. They are also sold along with other food materials. Smoked varieties are highly priced due to their desirable smoky flavour and attractive

appearance. Naini (*Cirrhinus mrigala*), Hilsa*, Garra gotyla,* Hile, Garai (*Channa* spp.)*,* Asla (*Schizothorax* spp.) etc*.* are available in the smoked forms. *Garra gotyla*, and *Schizothorax* are hill stream fishes adapted to the cold water with high velocity. The price of these smoked fishes ranges from rupees 200 to 500 per kg. These fishes, besides having good flavour also have esthetic values and occupy a special place in Newari food culture.

a. Sun dried fishes:

Fishes preserved by sun-drying are also preferred by people living in Kathmandu. Fishes like *Puntius sophore, Putnius ticto, Oxygaster* spp. etc comes in sundried forms. These fishes are also liked by costumers as they are best for pickles. People of Newar community consume a great deal of sun dried fish to add flavour in various dishes.

b. Salted fishes:

Salted fish include *Harpadon nehereus*, eel, etc. which are imported from India. These fishes add good flavour to the vegetable curries. The average price of these fishes ranges from Rs.200 to 250 per kg.

Different types of Preserved fishes found in Kathmandu, their rate per kg and their sources are listed in Table-3.

Table-3: List of different types of preserved fishes found in Kathmandu and their sources.

S.No.	Local Name	Scientific name	Type of preservation	Rate per kg	Source
1	Common carp	*Cyprinus carpio*	Chilling	Rs. 85-95	(Terai)
2	Bigheaded carp	*Aristichthys nobilis*	Chilling	Rs.125	Terai
3	Silver carp	*Hypothalmicthys molitrix*	Chilling	Rs.125	Terai
4	Rohu	*Labeo* spp.	Chilling	Rs.145	Terai
5	Naini	*Cirrhinus mrigala*	Chilling\ Smoking	Rs.100-125	Terai
6	Buhari	*Wallago attu*	Chilling	Rs. 145	Terai
7	Tengra	*Mystus* spp.	Chilling	Rs.200	India

8	Bam	*Macrognathus aculeatus*	Chilling	Rs.300	Terai
9	Singhi	*Heteropneustes fossilis*	Sun-drying	Rs.400	Terai
10	Sidre	*Puntius* spp**.**	Sun-drying	Rs.400	India
11	Chaluwa	*Oxygaster* spp.	Sun-drying	Rs.300	India
12	Asala	*Schizothorax* spp**.**	Smoking	Rs.400	Malekhu
13	Sahar	*Tor* spp.	Smoking	Rs.350	
14	Katle	*Neolissocheilus hexagonolepis*	Smoking	Rs.200	Terai
15	Buduna	*Garra gotyla*	Smoking	Rs.400	Malekhu
16	Bombay duck	*Harpadon nehereus*	Salted		India
17	Pomfert		Chilling		India
18	Jalkapoor	*Clupisoma garua*	Chilling	Rs. 200	Terai
19	Garae	*Channa* spp.	Smoking	Rs. 350	Terai and hills
20	Bhakur	*Catla catla*	Chilling		Terai

The price of preserved fishes has increased in a great deal in a last few decades due to their high demand and also due to the increase in the price of transportation fare. However people still prefer fish meat to other animal meat because fish meat is considered good for health. The seasons in which fishes are sold most include November, December, January and February.

Another type of preserved fish popular among the people of Kathmandu valley is canned fishes which are available in almost every grocery. These are imported from China, Thailand, and Malaysia etc. The cans come in different sizes but usually they are small and contain about 200gm of fish. They are available in different flavors. Some are preserved in plain brine while some are cooked in tomato sauce. Usually people of high living standard consume canned fish as they are rather expensive than other types of preserved fishes.

B. Microbial analysis:

I. Isolation of Bacteria:

The results of microbial analysis of Sun-dried, Salted and Smoked fish are given in Tables 4-10. Table-4 shows that Gram negative rods have been isolated from the head region of sun-dried fish. The colonies as seen on the culture plate were small rounded and yellow in colour. Bacteria isolated from the body region of sun-dried fish

were also Gram negative rods.

Table 4: Bacteria isolated from the head and body region of sun-dried fish. (*Oxygaster* spp)

S. No.	Sample	Dilution	No. of Colonies	Gram Stain	Morphological shape of
1.	Head	10^{-3}	7	Gram -ve	rods
2.	Head	10^{-2}	5	Gram -ve	rods
3.	Body	10^{-3}	TNTC	Gram-ve	rods in chain
4.	Body	10^{-2}	2	Gram -ve	rods

Table-5 represents the number and shape of bacteria isolated from head and body regions of salted fish *(Harpadon nehereus*). Gram negative rods have been isolated from these regions of salted fish.

Table-5: Bacteria isolated from the head and body region of salted fish (*Harpadon nehereus*)

S. No.	Sample	Dilution	No. of Colonies	Gram Stain	Morphological shape of bacteria
1.	Head	10^{-3}	3	Gram-ve	Rods
2.	Head	10^{-2}	5	Gram-ve	Rods
3.	Body	10^{-3}	TNTC	Gram-ve	Rods
4.	Body	10^{-2}	70	Gram-ve	Rods

Table-6 represents the bacterial species isolated from the head and body regions of Smoked fish (*Cirrihinus mrigala*). Both Gram negative and Gram positive bacteria have been isolated from this region. The Gram positive bacteria as seen under microscope were cocci where Gram positive bacteria were rods.

Table-6: Bacteria isolated from the head and body regions of smoked fish (*Cirrhinus mrigala*)

S.No	Sample	Dilution	No. of Colonies	Gram stain	Morphological shape of bacteria
1.	Head	10^{-1}	TNTC, 50	Gram -ve & Gram +ve	a. Rods b. Cocci
2.	Head	10^{-2}	50	Gram +ve	Cocci
3.	Body	10^{-1}	20	Gram +ve	Cocci
4.	Body	10^{-2}	TNTC	Gram +ve	Cocci

From above tables we can conclude that the number of bacteria isolated from head region is more than those isolated from body region in all three cases. Gram stain results show that Gram negative bacteria has occurred in more number than Gram positive bacteria.

i. Identification of Gram negative bacteria:

To identify Gram negative bacteria it was necessary to perform **Biochemical Tests**. Table-7 shows the results of biochemical tests performed to identify Gram negative bacteria isolated from the head and body regions of sun-dried fish (*Oxygaster* spp.) The bacteria isolated from head region were identified as *Escherichia coli* and *Salmonella* spp. while from body region, *Salmonella* spp. and *Salmonella paratyphi* A were isolated.

Table-7: Result of biochemical tests performed to identify Gram-ve bacteria isolated from sun-dried fish (*Oxygaster* spp.)

Test/S. No.	1	2	3	4
Dilution	Head, 10^{-3}	Head, 10^{-2}	Body, 10^{-3}	Body, 10^{-2}
SIM Test	Sulphide –ve Indole-ve Motility –ve	Sulphide –ve Indole –ve Motility+ve	Sulphide –ve Indole –ve Motility +ve	Sulphide –ve Indole –ve Motility+ve
TSI Test	Gas +ve Pink colour	Alkaline\alkaline Gas +ve R\Y	Alkaline\alkaline Gas –ve R\Y	Alkaline\alkaline Gas –ve R\Y
MR	-ve	+ve	+ve	+ve
VP	+ve	-ve	-ve	-ve
Urease test	-ve	-ve	-ve	-ve
Citrate test	+ve	+ve	+ve	+ve
OF test	Acid (yellow)	Acid (yellow)	Acid (yellow)	Acid (yellow)
Catalase test	Bubble formation +ve	Bubble formation +ve	Bubble formation +ve	Bubble formation +ve
Bacteria	*Escherichia coli*	*Salmonella* spp	*Salmonella* spp	*Salmonella* *paratyphi* A

Table 8 shows the results of biochemical tests performed on the Gram negative bacteria isolated from the head and body regions of salted fish (*Harpadon nehereus*). They were identified as *Enterobacter* spp. and *Salmonella* spp. respectively.

Table 8: Result of biochemical tests performed on Gram-ve bacteria isolated from salted fish (*Harpadon nehereus*).

Test/S. No.	1	2	3	4
Dilution	Head, 10^{-3}	Head, 10^{-2}	Body, 10^{-3}	Body, 10^{-2}
SIM Test	Sulphide –ve Indole –ve Motility +ve	Sulphide –ve Indole –ve Motility+ve	Sulphide –ve Indole –ve Motility+ve	Sulphide –ve Indole –ve Motility+ve
TSI Test	Gas+ve H_2S –ve Acid\Acid Y\Y	Gas +ve Alkaline\ Acid R\Y	Gas +ve H_2S –ve Acid\Acid Y\Y	Gas +ve Alkaline\ Acid R\Y
MR	-ve	+ve	-ve	+ve
VP	+ve	-ve	+ve	-ve
Urease test	-ve	-ve	-ve	-ve
Citrate test	+ve	+ve	+ve	+ve
OF test	Acid (yellow)	Acid (yellow)	Acid (yellow)	Acid (yellow)
Catalase test	Bubble formation +ve	Bubble formation +ve	Bubble formation +ve	Bubble formation +ve
Bacteria	*Enterobacter* spp.	*Salmonella* spp.	*Enterobacter* spp.	*Salmonella* spp.

Table-9 gives the results of biochemical tests performed to identify Gram negative bacteria isolated from the head region of smoked fish (*Cirrhinus mrigala*). The bacteria were identified as *Pseudomonas* spp.

Table-9: Result of biochemical tests performed on Gram-ve bacteria isolated from smoked fish (*Cirrhinus mrigala*).

Test/S. No.	1
Dilution	Head, 10^{-2}
SIM Test	Sulphide –ve Indole –ve Motility +ve
TSI Test	Gas –ve H_2S –ve Acid\Acid Y\Y
MR	-ve
VP	+ve
Urease test	-ve
Citrate test	+ve
OF test	Acid (yellow)
Catalase test	Bubble formation +ve
Bacteria	*Pseudomonas* spp.

ii. Identification of Gram Positive Bacteria:

Positive results of Gram stain were given by bacteria isolated from head and body regions of smoked fish (*Cirrhinus mrigala*) only. Table 10 shows the species of Gram positive bacteria isolated from the head and body regions. In head region *Micrococcus* and *Staphylococcus* spp. were isolated while from

body region only *Micrococcus* is isolated.

Table-10: Gram positive bacteria isolated from the head and body regions of smoked fish (*Cirrhinus mrigala*)

S.No	Sample	Dilutions	Gram stain	Bacteria
1	Head	10^{-1}	Gram+ve cocci	*Micrococcus*
2	Head	10^{-2}	Gram+ve Cocci	*Staphylococcus* spp.
3	Body	10^{-1}	Gram+ve Cocci	*Micrococcus*
4	Body	10^{-2}	Gram+ve Cocci	*Micrococcus*

III. Isolation of Fungi

Table 11 shows the species of fungi isolated from the head and body regions of salted and sun-dried fish respectively. The fungal species isolated from the head and body regions of salted fish are *Trichoderma* spp. The colony of *Trichoderma* was cottony white in appearance. Similarly, the fungal species isolated in sun-dried fish are *Trichoderma* spp. and *Aspregillus fumigatus.* The colony of *Trichoderma* was cottony white whereas that of *Aspergillus fumigatus* was green.

Table-11: Fungi isolated from the head and body regions of salted fish (*Harpadon nehereus*) and sun-dried fish (*Oxygaster* spp.)

S.No	Samples	Dilutions	Name of fungi isolated
1.	Salted fish, Head	10^{-2}	*Trichoderma* spp.
2.	Salted fish, Body	10^{-3}	*Trichoderma* spp.
3.	Sun-dried fish, Head	10^{-3}	*Aspergillus fumigatus*
4.	Sun-dried fish, Body	10^{-3}	*Trichoderma* spp.

Fungi were not isolated from smoked fish.

B. Chemical Analysis:

The chemical analysis of smoked, salted and sun-dried fish gave following results:

Table 12 shows that, the protein content of *Cirrhinus mrigala* (smoked) is 70.78 percent whereas that of *Harpadon nehereus* (salted) is 62.15 percent. Similarly the protein content of *Oxygaster* (sun-dried) was found to be 64.93 percent.

Table-12: Protein Content in Smoked, Salted and Sun-dried fish:

S.No.	Name Of Fish	Type of Preservation	Protein Content (wet basis)%
1.	*Cirrhinus mrigala*	Smoked	70.78
2.	*Harpadon nehereus*	Salted	62.15
3.	*Oxygaster* spp.	Sun-dried	64.93

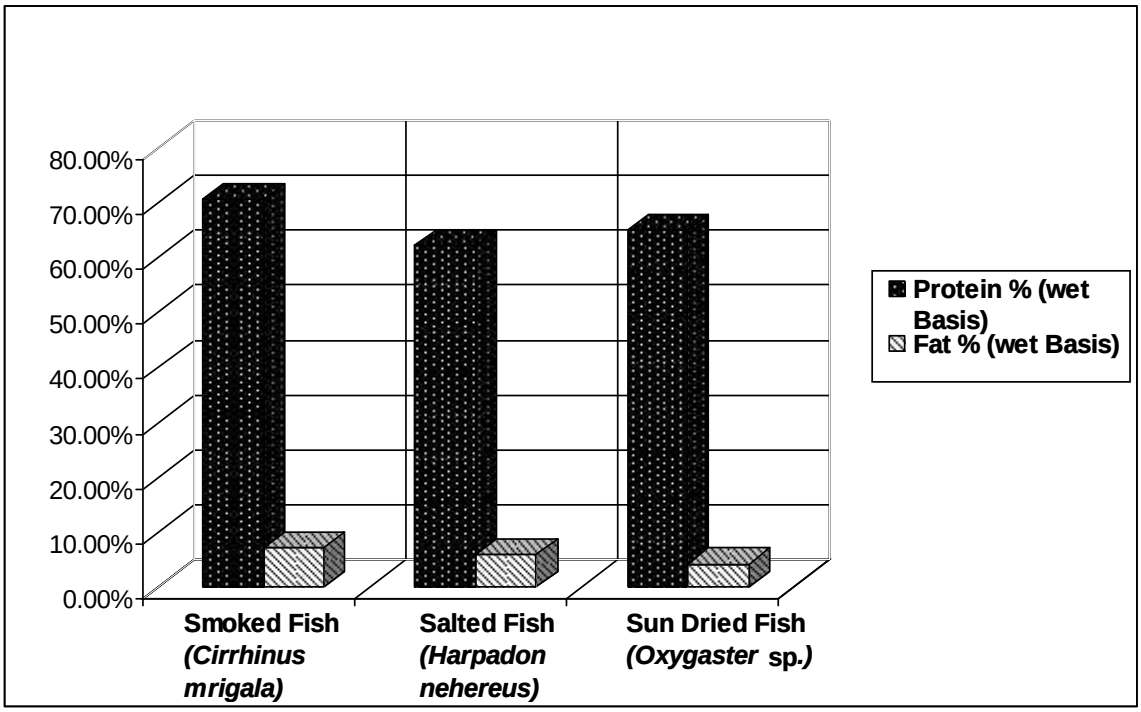
The fat content of fish flesh is the factor that determines the quality of fish and hence its price. From table 13 it becomes quite clear that the fat content of smoked fish, *Cirrhinus mrigala* (smoked) is highest among the three. It is about 7.23 percent whereas that of *Harpadon nehereus* and *Oxygaster* spp. is 6.03 and 4.21 respectively. Hence from these data we can conclude that the price as well as quality of *Cirrhinus mrigala* is higher than rest two.

Table-13: Fat Content in Smoked, Salted and Sun-dried fish

S.No.	Name Of Fish	Type of Preservation	Fat content (wet basis)%
1.	*Cirrhinus mrigala*	Smoked	7.23
2.	*Harpadon nehereus*	Salted	6.03
3.	*Oxygaster* spp.	Sun-dried	4.21

Below is the graphical representation of protein and fat percentage in smoked, salted and sun-dried fishes respectively.

Fig 3: Protein and fat percentage in smoked, salted and sun-dried fish



CHAPTER VI

DISCUSSION

The demand of fresh as well as preserved fish is quite high in the markets of Kathmandu. Thus there are plenty big and small shops scattered all over the valley that sell fish. Some of the famous areas for selling fish are Kichapokari, Kalimati and Asan of Kathmanu and Lagankhel and Mangal Bazaar of Latipur. Of these, preserved fish were sold mainly in Asan and Mangal Bazaar. There were many shops around Asan that sell different types of fish in preserved form. Beside these, many small and big groceries also sell different types of preserved fish along with other food stuffs.

Preserved fish are available in different forms, viz; chilled, salted, sun-dried and smoked. Fishes like Rohu (*Labeo* spp.), *Catla catla*, Buhari *(Wallago attu),* and *Channa* spp. Mungri (*Clarius batracus*), JalKapoor (*Clupisoma gaura*), Naini (*Crihhinus mrigal),* Sahar (*Tor* spp.) etc are available in chilled forms. Salted fish include *Harpadon nehereus*, sun-dried fish include *Puntius* spp., *Oxygaster* spp. etc. and smoked fish include Naini, *Schizothorax* spp., *Tor tor* etc. Most of these fish are supplied from different parts of India such as Andra Pradesh, Bombay while some are brought from different parts of Nepal as well.

It is well known that fish spoilage is primarily due to (i) autolysis, (ii) bacterial growth and metabolism resulting in the formation of off-flavor compounds, and (iii) chemical oxidation of lipids. Among these, microbiological activity is by far the most important factor influencing fish quality (Tryfinopoulou *et al.* 2001). Spoilage results in considerable annual economic loss to businesses and individuals (Anonymous, 1993). There are several reasons that can lead to such microbiological spoilage. One of such reasons is the hygienic condition of the shops that sell these fish which are not quite satisfactory in Kathmandu. Often they are seen being sold on the pavements or along the streets. This can be an invitation to the pathogenic bacteria to invade upon the fish.

Micro-flora contamination also depends upon the personal hygiene of people engaged in the preservation process. If the hygienic condition of people concerned with preservation process is poor, then such products have more chances of being

contaminated. Another important reason for bacterial contamination is improper sanitation. Most of the pathogenic bacteria found on fish meat are due to poor sanitation and rough handling by the shopkeepers.

According to the survey work undertaken here the hygienic condition of fish markets of Kathmandu was found to be quite poor. There has been no considerable improvement in the hygienic condition of markets for nearly two decades. Chaudhary (1989) in his research work has reported the poor hygienic condition of fish markets of Kathmandu. Then, the fishes were sold along the pavement and separate fish market was lacking. According to the present survey, a separate fish market has been opened at Kalimati where there are several shops that sell fishes in chilled conditions. However, due to the lack of good quality water, proper sanitation, unavailability of plenty of chilled rooms, refrigerator, containers etc. have made the fishes susceptible to microbial contamination.

The laboratory work undertaken here to study the micro-flora of preserved fish available in the market of Kathmandu revealed the presence of different pathogenic bacteria and fungi harmful to mankind if not cooked well before consuming. No such studies have been made yet in fishes available locally in Kathmandu market.

However, Chaudhary (1989) isolated different types of microbial organisms in three different types of fish viz., *Labeo rohita, Harpadon nehereus, Puntius sophore* and *Schizothorax plagiostomus* selected as representatives of fresh, salted, sun-dried and smoked fishes respectively. His result showed the frequent occurrence of Gram-negative bacteria in all types of fishes. The bacterial colonies which he isolated from *Schizothorax plagiostomus* were found to be *Pseudomonas*, *Leuconostoc*, *Micrococcus* and *Escherichia coli*. In case of salted fish (*Harpadon nehereus*), he isolated *Pseudomonas, Leuconostoc, Micrococcus* and *Lactobascilus* .The bacterial colonies which he isolated from *Puntius sophore* (sun-dried) were *Pseudomonas, Micrococcus, Leuconostoc* and *Escherchia coli*. In the present work, bacteria such as *Escherichia coli* and *Salmonella* spp. were isolated from the sun-dried fish (*Oxygaster* spp.). The bacterial colonies isolated from smoked fish (*Cirrhinus mrigala*) in this present work are *Pseudomonas, Micrococcus* and *Staphylococcus.* Similarly, in salted fish (*Harpadon nehereus*), bacteria such as *Enterobacter* and *Salmonella* spp. were isolated.

The initial flora on fish is very diverse, although most often dominated by Gram-negative psychotropic bacteria. Fish caught in tropical areas, may carry a slightly

higher load of Gram-positive organisms and enteric bacteria. Pathogenic bacteria that usually occur in fish include bacteria belonging to Enterobacteriaceae species such as *Salmonella, Shigella, Escherichia coli* etc. and *Pseudomonas*, *Staphylococcus aureus* etc. (Anonymous, 1998).

***Salmonella* spp.**

These are the mesophilic organisms, distributed all over the world, but principally occurring in the gut of man and animals and in environments polluted with human or animal excreta. Thus the contamination by *Salmonella* indicates the matter of poor sanitation. They cause Salmonellosis (non-typhoid infections). The principal symptoms of this disease are non-bloody diarrhea, abdominal pain, fever, nausea, vomiting which generally appear 12-36 hours after ingestion. The disease may also proceed to serious complications.

Staphylococcus aureus

Staphylococcus is a Gram-positive bacterium. The natural reservoir of *S. aureus* is human skin, hair and superficial mucous membranes, while it is not part of the normal flora on fish and fish products. Contamination with *Staphylococcus* is via infected food handlers or from the environment. More often the contamination is from an individual with an infection on hands or with a cold sore throat. It is mesophilic with minimum growth temperature of 10^{o}C, but higher temperatures are required for toxin production. Presence of *S. aureus* in large number indicates the possible presence of enterotoxin and\or faulty sanitary or production practice. *Staphylococci* are poor competitors and do not grow well in the presence of other microorganisms. Thus the presence of *Staphylococci* in raw, naturally contaminating food is of little significance. In contrast rapid growth and toxin production can take place in precooked fish. *S. aureus* produces a number of enterotoxins, when growing in the food. These toxins are generally very resistant to proteolytic enzymes and heat.

Escherichia coli

The natural habitat of *Escherichia coli* is the intestine of human and vertebrate animals. In temperate water this organism is absent from fish at the time of capture. This organism is therefore particularly useful as indicator of contamination or mishandling such as temperature abuse in product handling (Huss, 1994).

***Pseudomonas* spp.**

Pseudomonas is a Gram-negative, aerobic rod belonging to the bacterial family *Pseudomonadaceae.* These bacteria are common inhabitants of soil and water. They occur regularly on the surfaces of plants and occasionally on the surfaces of animals (Todar, 2004). Due to their ability to utilize a wide range of organic compounds, they occupy an important ecological position in the carbon cycle. Therefore, the ecology of pseudomonads in the biosphere has been a matter of interest. In this sense, an accurate system for the classification and identification of pseudomonads is an essential prerequisite for a detailed investigation of the roles and evolution of these bacteria originating in either animal (e.g., fish and meat) or plant products.

In the current work, Gram positive cocci such as *Staphylococcus and Micrococcus* were isolated from smoked fish. Erichsen (1966), also isolated *Micrococcus* and *Staphylococcus in* semi-preserved fish. Similarly, Gonzalez *et al.* (2002), isolated Enterobacteriaceae, lactic acid bacteria (LAB), fungi and presumptive aeromonads and staphylococci in their analysis performed on smoked fish. *Proteus vulgaris*, *P. mirabilis* and *Serratia liquefaciens* were dominant among Enterobacteriaceae and coagulase-negative staphylococci among Micrococcaceae.

According to the literature review, micro-floral contamination is more prevalent in smoked, salted and sun-dried fishes than chilled fishes. Chilling method of preservation is more sophisticated and hygienic than smoking, salting and sun-drying methods. Thus fishes preserved by this method are less likely to undergo micro-floral contamination

Kazanas (1966), on his research on yellow perch fillets isolated *Alcaligenes*, *Pseudomonas*, *Micrococcus*, *Bacillus*, *Sarcina*, yeasts, *Lactobacillus, Aeromonas*, and a few *Proteus* and *Escherichia* cells. In this present work also *Pseudomonas* and *Micrococcus* were isolated from the head and body regions of smoked fish respectively.

In this present work, species of *Salmonella and Staphylococci* were isolated. Species of *Salmonella* occurred in sun-dried (*Oxygaster* spp.) and salted fish (*Harapdon nehereus*) respectively, whereas *Staphylococci* occurred in smoked fish (*Cirrhinus mrigala*). Nauki in 1975 examined Atlantic mackerel (*Scomber scomburs*) both its skin surface and the musculature prior to and after thawing, cleaning and washing and salting. Micro-flora such as coliforms, bacilli, moulds, yeasts, *Salmonellae* and

pathogenic *Staphylococci* were isolated. It was found that during processing the microbial contamination of fish considerably rises.

Species of *Pseudomonas* were isolated from the smoked fish (*Cirrhinus mrigala*) in this current study. Tryfinopoulou *et al*. (2002), worked together on gilt-head sea bream Mediterranean fish (*Sparus aurata*) stored under different conditions. They isolated different strains of *Pseudomonas* from every sample. Similarly, Koutsoumanis (2001), worked on Gilt-head Sea bream (*Sparus aurata*) and isolated Pseudomonads which were established as the main bacterial contamination *Sparus aurata.*

According to the research covered on qualitative and quantitative analyses of micro flora of traditionally and vacuum-packed low-salt herring fillets by Dąbrowski *et al.* (2001), bacteria such as *Micrococcus* spp. and *Staphylococcus* spp. were isolated. Gram-negative rods such as *Pseudomonas* spp*.* and *Xanthomonas* spp. were also isolated In the latter *Escherichia* spp*.* was also found. *Escherichia coli* were also isolated in present work from sun-dried fish. The colonies of *E. coli* as observed on the (NA) Nutrient Agar were small rounded and yellow in colour.

In present work, besides the culture of bacteria, fungal culture was also performed to the same samples of fishes. It has been found that the fungal spoilage of fish is comparatively low than the bacterial spoilage. According to Lowery (1984) yeast and mould are usually thought to play merely a minor role in the spoilage of fish because they constitute only a small portion of the initial micro flora and because they grow slowly compared with most bacteria. The small growing molds can spoilage fish by forming visible colonies but this occurs only when storage conditions selectively inhibit bacterial development.

According to Frazier and Westhoff (1978) have pointed out that molds are the chief spoilage organisms on smoked fishes. The incipient growth of molds makes the surface of the fish sticky to touch. It gives a musty flavour to fish in the vicinity of their growth.

Chaudhary (1989) also isolated the number of yeast and mold colonies from chilled and dried fishes. However, he had not specified the species of yeast and moulds which he isolated in his research.

Shrestha (1989) has studied the role of fungal flora in buff meat contamination and

spoilage. She isolated a number of fungus such as *Penicillium* spp. *Aspergillus* spp. Yeasts, *Alternaria* etc. She has also reported the effect of pH and water activity on fungal deterioration of buff.

In the present work, species of fungi such as *Aspergillus fumigatus* and *Trichoderma* spp. have been isolated from salted and sun-dried fish. In case of smoked fish the fungal growth was absent.

Aspergillus fumigatus was isolated from sun-dried fish in present work. The colony of *A. fumigatus* that grew on the PDA (Potato Dextrose Agar) plate was green in colour. Atapattu and Samarajeewa (1989), studied the mycoflora of dried-salted fish. They isolated a total of 61 fungal from 25 dried-fish and identified them. Their research showed that the most prevalent fungus was *Aspergillus niger*. Species of *Aspergillus flavus, A. fumigatus, A. glaucus, A. restrictus, Aureobasidium* spp. *Gliomastix*, spp., *Penicillium chalybeum* and *Penicillium expansum* were present. Similarly, Fafioye *et al.* (2002) while working on different types of smoked fishes isolated fungi such as *Mucor* spp., *Aspergillus* spp., *Rhizopus* spp. and *Fusarium* spp.

According to the literatures found so far, *Aspergillus* spp. have been found to be most frequently occurring in dried fishes. *Aspergillus* is a filamentous, cosmopolitan and ubiquitous fungus found in nature. It is commonly isolated from soil, plant debris, and indoor air environment. The genus *Aspergillus* includes over 185 species. Around 20 species have so far been reported as causative agents of opportunistic infections in man. Among these, *Aspergillus fumigatus* is the most commonly isolated species. In the present work also *Aspergillus fumigatus* was isolated from the dried fish. *A. fumigatus* produces toxic substance which causes allergic diseases in asthmatics and patients suffering from cystic fibrosis. Invasive aspergillosis can occur in individuals with cavities caused by tuberculosis or other cystic lung diseases.

The bacteria analysis of fishes selected for this work showed that the isolation of bacteria belonging to Enterobateriaceae species was more in number. The members of this bacterium are Gram negative, straight rods, non-motile or motile with peritrichous flagella, occurring in intestines and on other substrates also. Bacteria such as *Escherichia coli*, *Salmonella* spp. *Enterobacter* spp. were isolated from salted (*Harpadon nehereus*) and sun-dried (*Oxygaster* spp.) fishes in this work. In case of smoked fish (*Cirrhinus mrigala*) both Gram-negative and Gram-positive bacteria are isolated. A Gram-negative bacterium isolated from smoked fish was *Pseudomonas*. It has 90 species, occurring on diverse habitats such as soil, food

materials and mouth parts of man and animals. The cells are straight, slightly curved rods with one or more polar flagella. *Micrococcous* and *Staphylococcus* are the Gram-positive bacteria isolated from smoked fish.

Chaudhary (1989) also isolated *Micrococcous*, *Pseudomnas* and *Escherichia coli* from smoked, salted and sun-dried fishes. But the species of fish from which he isolated these bacteria are different than those of the present work. For instance, in present work (*Cirrhinus mrigala*) was selected as the representative of smoked fish whereas in his work *Schizothorax plagiostomus* was selected as the representative of smoked fish. In present work, *Pseudomonas, Micrococcus* and *Staphylococcus* were isolated from smoked fish whereas in his work, *Pseudomonas*, *Leuconostoc*, *Micrococcus* and *Escherichia coli* were isolated from smoked fish. In present work *Salmonella* spp. and *Enterobacter* spp. were isolated from salted fish (*Harpadon nehereus*). Although Chaudhary (1989) worked on the salted fish of same species however he isolated *Pseudomonas, Leuconostoc, Micrococcus* and *Lactobascilus* which are different than the ones isolated from the present work.

In present work *Oxygaster* spp. was selected as the representative of sun- dried fish. From this fish bacteria such as *Escherchia coli* and *Salmonella* spp. were isolated from it. Chaudhary (1989) in his research selected *Puntius sophore* as the representative of sun-dried fish. From the culture of bacteria he isolated *Pseudomonas, Micrococcus, Leuconostoc* and *Escherchia coli.* Even though present work is similar to the one undertaken by Chaudhary (1989), the results obtained from the two works are comparatively different. The occurance of difference is due to the difference in fish species selected in two works except for salted fish (*Harpadon nehereus*).

In the present work, the fungal analysis showed the occurrence of *Aspergillus fumigatus* and *Trichoderma* spp.. Chaudhary (1989) have also isolated yeast and molds from all three types of dried fishes. However, he has not specified the species name.

There are no published references available regarding the fungal and bacterial contents of the fish in Nepal so far besides those of Chaudhary (1989).

An attempt has also been made in the present work to study the chemical composition of preserved fish. Thus, the chemical content such as protein and fat were estimated. The protein content of smoked fish (*Cirrihinus mrigala*) was 70.78%,

the protein content of salted fish (*Harpadon nehereus*) was 62.15% and that of sun-dried fish (*Oxygaster* spp.) was about 64.93%. (Table V). The fat content of smoked, salted and sun-dried fish was 7.23%, 6.03% and 4.21% respectively (Table V).

Several scientists have worked on the chemical composition of fishes. Foda *et al*. (1986) studied the chemical constituents of Bolte fish and reported that small fishes contain higher percentage of waste protein in relation to larger one. Lee (1987) studied the lipid composition and seasonal variation in fatty acid composition of body oil in several fishes.

Chaudhary (1989) also estimated the amount of protein, fat and other minerals of three different types of preserved fish. His work showed that the percentage of protein and fat is highest in case of smoked fish than in case of sun-dried and salted fish. The protein content of smoked fish (*Schizothorax plagiostomus*) was about 60.31 percent, that of salted fish (*Harpadon nehereus*) was 59.03 percent and the protein content of sun-dried fish (*Puntius sophore*) was 59.67 percent. Similary, the fat content of smoked fish was about 7.86 percent, that of salted fish was about 8.33 percent and the fat content of sun-dried fish was 8.00 percent.

The biochemical analysis of fishes selected for this study showed that the protein content is highest (70.78%) in smoked fish followed by sun-dried fish (64.93%) and the lowest in salted fish (62.15%). The fat content also has been found to be highest (7.23%) in smoked fish but lowest (4.21%) in sun-dried fish. In salted ones it was 6.03 % which stands between smoked and sun-dried ones.

The trend of protein content in smoked, salted and sun-dried fishes in Chaudhary's observations is more or less similar to those found in the present work while in fat content the trend has been found different. In his case, fat content is highest in salted fish followed by sun-dried fish and the lowest in smoked fish. Not necessarily, these differences are due to the difference in type of preservation. It may be due to the difference in species selected for the study. Further works towards this direction will confirm past records and the present finding.

In present work, large numbers of bacterium were isolated from smoked fish which also has more protein and fat percentages than the salted and sun-dried fish.

CHAPTER VII

CONCLUSION

There has been no considerable improvement in the hygienic condition of fish markets of Kathmandu since past ten, fifteen years. Separate fish markets are still lacking and fishes are sold in very congested market areas where they are easily invaded by micro-flora.

Micro-flora such as bacteria and fungi contaminate fish making them unfit for human consumption. Rough handling by those involved in selling of fish and the poor hygienic condition of fish shops are the main factors which lead towards micro floral contamination. In the present work both bacteria and fungi have been isolated from all three different types of preserved fish.

The results of bacterial culture have revealed that Gram negative rods, belonging to Enterobacteriaceae have prevailed over others. Species of Enterobacteriaceae includes *Salmonella, Escherichia coli, Enterobacter* etc. In salted fish (*Harpadon nehereus*), colonies of Enterobacteriaceae spp. such as *Salmonella* spp. and *Enterobacter* were isolated. Similary, in sun-dried fish (*Oxygaster* spp.) also species of *Salmonella* and *Escherichia coli* were isolated. In the present work, bacteria such as *Pseudomonas, Micrococcus* and *Staphylococcus* were isolated from smoked fish (*Cirrhinus mrigala*). These bacteria are quite pathogenic and cause health problems.

Besides bacterial culture the preserved fish were also subjected for fungal culture. Two different types of fungal conlonies were isolated. In sun-dried fish colonies of *Trichoderma* spp. and *Aspergillus fumigatus* were isolated. In case of salted fish colonies of *Trichoderma* spp. were isolated.

Thus an attempt was also made in the present work to study about chemical content such as protein and fat. The percentage of crude protein was determined by estimating the total nitrogen by Kjeldahl method in wet basis. Similarly, for the extraction of crude fat, Soxlet Extractor Method was used which involved the extraction of fat by distillation with organic solvent. The protein content of smoked fish (*Cirrihinus mrigala*) was 70.78%, the protein content of salted fish (*Harpadon*

nehereus) was 62.15% and that of sun-dried fish (*Oxygaster* spp.) was about 64.93%. The fat content of smoked, salted and sun-dried fish was 7.23%, 6.03% and 4.21% respectively.

CHAPTER VIII

RECOMMENDATION

- The nutritional value of fish is very high and addition of fish meat in diet can be very beneficial for people of all ages. However fish meat is highly perishable and lack of proper preservation can result in the loss of quality as well as quantity of fish meat. So to avoid such loss there must be proper preservation system. Modern preservation systems like freezing, canning must be implemented in those parts of our country where there is a commercial catch of fish.

- The hygienic condition of fish markets in Kathmandu must be improved. There must be a separate fish market instead of selling them on pavements in market areas. The physical facilities like quality of tap water, cold storage, preservatives and sanitary condition should be improved in the fish market. Selling of fish along on the pavements should not be encouraged.

- The microbial as well as biochemical studies of fish should be made as often as possible by concerning institutes.

- It has been found that these bacteria and fungi are killed at the 120^{o}C. Thus it is necessary to make sure that these fishes are well-cooked before consuming. The majority of the micro flora that were isolated is pathogenic and can cause serious health problems. However, if properly cooked, these fish are safe enough to consume as the micro flora present on them are already killed by the heat.

- A system of checking the hygienic condition of fish should be developed before reaching to the fish consumers.

- The analysis the moisture content of fish may be performed in further studies.

REFERENCES

Anonymous, (1993). Foiling Spoilage with Chemical Preservatives Kit. https://www2.carolina.com/webapp/wcs/stores/servlet/ProductDisplay?jdeAddressId=&catalogId=10101&storeId=10151&productId=2265&langId=-1&parent_category_rn=83%7C1696%7C1737

Anonymous, (1962). The Wealth of India. A Dictionary of Indian Raw materials and Industrial Products. Raw materials Vol. iv, Supplement Fish and Fishries. Council of scientific & industrial research New Delhi, pp. 96-105.

Anonymous, (1998). The State of World Fisheries and Aquaculture, FAO.

Anonymous, (2004). The State of World Fisheries and Aquaculture, FAO.

Anonymous, (2006). *Aspergillus fumigatus* Genome Project www.sanger.ac.uk/Projects/A_fumigatus

Atapattu R. and Samarajeewa U., (1990): Mycopathologia. Fungi associated with dried fish in Sri Lanka, Volume 111,Number1\July,10.1007/BF02277304, 55-59.

Bajracharya D., (1999). Experiments in plant physiology. A Laboratory Manual. Narosa Publishing House. 6 Community Centre, Panchsheel Park, New delhi 110 017.

Bhattrai S., (2001): Study on Micro flora of Vermicompost and its Antagonistic Activity against Plant Pathogenic Bacteria. Central department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu.

Brian A., (2006). The Bacterial Microflora of Fish, The Scientific World Journal, vol. 6. pp. 931-945.

Chaudhary R. P., (1989). Study on the Preservation and Marketing of Fishes of Kathmandu with special reference to their Microbiology and Chemical Analysis, M.Sc. Dissertation, Tribhuvan University, Kirtipur, Kathmandu.

Cheesbrough M., (1894). Medical Laboratory Manual for tropical Countries, Vol.II: Microbiology 1st edn, ELBS, Cambridge University Press.

Dąbrowski W., Czeszejko K., Gronet A., and Wesołowska A., (2001). Micro flora of low-salt herring I. The effect of sort packing on micro flora of herring A‘la matias. Volume 4, Issue 2, Series: food and science technology.

Dahal, S.P., (1998). An observation on fresh fish marketing in Kathmandu Valley. Presented at the Workshop for the Natural Water Project, Pokhara, July 3-4, 1998. 10pp.

Erichsen I., (1966). Swedish Institute for Food Preservation Research, Göteborg, Sweden 3 May.

Fafioye O.O.Â, Efuntoye M.O.Â and Osho A.Â, (2002). Studies on the fungal infestation of five traditionally smoke-dried freshwater fish in Ago-Iwoye, Nigeria. Vol.154, Number 4\August, 10.1023/A: 1016331418893, 177-179.

Fankhauser D.B. (2005), Agar overlay technique. http://biology.clc.uc.edu/fankhauser/Labs/Microbiology/Bacterial_Inhibition/Agar_Overlay.htm

Foda U.H., Ferial, N., Abusalem, S., Dashloutynagwa, E.L., Ramsy, M and Sorore, AFAF, (1986). Chemical Constituents of Bolte Fish, A.M.N. Agri. Science Cairo 31(1) 499-518 Vol. 83.

Frazier W.C. and Westhoff D.C., (1978). Food Microbiology, Tata Mc Graw-Hill Publishing Company Limited C-61 Mayapuri 11-New Delhi.

Gonzalez-Rodriguez M.N, Sanz J.J., Santos J.A., Otero A. and Garcia-Lopez M.L. (2002). Numbers and types of microorganisms in vacuum-packed cold-smoked freshwater fish at the retail level. http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=Retrieve&db=PubMed&list_uids=12076034&dopt=Abstract

Gram L. and Huss H.H., (1996). Microbiological spoilage of fish and fish products, http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=Retrieve&db=PubMed&list_uids=8

913813&dopt=Abstract

Huss H.H., (1994). Assurance of Seafood quality, FAO Fisheries Technical Paper 334.

Kazanas N., (1966): Effect of γ Irradiation on the Micro flora of Freshwater Fish: II. Generic Identification of Aerobic Bacteria from Yellow Perch Fillets. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1058449

K.C. J. Ram., (1999): The Fresh water Fishes of Indian region, Narendra Publishing House, India, 551.

Khanna S. S., (1970). An Introduction to Fishes, Central Book Dept., Indian University Press, Allahabad.

Knochel S. and Huss H H. (1984): Ripening and Spoilage of sugar salted herring with and without nitrate, Journal of Food Technology, 19 (2), 203–213.

Koutsoumanis K., (2001). Predictive Modeling of the Shelf Life of Fish under Nonisothermal Conditions**.** Laboratory of Microbiology and Biotechnology of Foods, Department of Food Science and Technology, Agricultural University of Athens, Athens 11855, Greece.

Leekang N., Inhak Jeong, Jae-SOO-suh, Woon-Jin Jung Chung Gon Kim and Byeong-Ho Lee, (1987): Lipid Composition and seasonal Variation in Fatty Acid Composition of Body Oil. Department of Food Sci. Technol. Korea.

Lowery, P.D., (1984): Limiting Conditions for Yeast Growth on Frozen Meat. Bacterial 56(2): 193-200.

Medeiros L.C., (2006): Home Preservation of Fish, HYG-5331-91, Ohio State University Extension Fact Sheet, Human Nutrition and Food Management http://ohioline.osu.edu/hyg-fact/5000/5331.html

Nauki V.M., (1975): Bacterial contamination of salted ocean mackerel during the production process. 12(2):51-7. http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=Retrieve&db=PubMed&list_uids=1129940&dopt=Abstract

RAO. A.S., (1997). Introduction to Microbiology. Prentice-Hall of India Private Limited, New Delhi.

Riches D., (2007). Your Complete Guide to Smoking Fish, Your Guide to Barbecues and Grilling.
http://bbq.about.com/cs/fish/a/aa030400a.htm

Sasson A., (1990). Feeding Tomorrow's World.Paris, UNESCO/CTA, 805pp

Shrestha J., (1981). Fishes of Nepal. Curr. Dev. Cent. Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Shrestha, J. (1994). Fishes, fishing implements and methods of Nepal. Smt. M.D. Gupta, Ladetpur Colony Lashkar, India. 145pp.

Shrestha J., (1995). Enumeration of Fishes of Nepal. Biodiversity profiles Project, HMG of Nepal\Government of Netherlands, Kathmandu, Nepal.

Shrestha J., (2001). Taxonomic Revision of Fishes of Nepal, Environment and Agricultural Biodiversity, Agriculture and Pollution in South Asia. Published by Ecological Society (ECOS), P.O.6132, Kathmandu, Nepal, pp. 171-180.

Shrestha J., (2003). Taxonomic Revision of Cold Water Fishes of Nepal. Cold Water Fishes in the Trans. Himalayan Countries. FAO. Fisheries technical Paper 431. pp. 273-288.

Shrestha S.G., (1989). The Role of Fungal Flora in Buff Meat Contamination ans Spoilage, M.Sc. Dissertation, Tribhuvan University, Kirtipur, Kathmandu.

Shrestha T.B., (1999). Nepal Country Report on Biological Diversity Kathmandu: IUCN Nepal. Ix, 133pp.

Srivastava, C.B.L., (1985): text Book of Fishery Science and Indian Fisheries, Kitab mahal 15, Thornhill Road, Allahabad, India.

Thiel T., (1999). Nutrient Broth, Agar Plates and Slants, Science In real World: Microbes in Action.

Todar K., (2004). Todar's Online Text Book of Biology, University of Wisconsin-Madison, Department of Bacteriology. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1366868

Tryfinopoulou P., Tsakalidou E., and Nychas G. J.E., (2002). Characterization of *Pseudomonas* spp. Associated with Spoilage of Gilt-Head Sea Bream Stored under Various Conditions. http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=Retrieve&db=pubmed&dopt=Abstract&list_uids=11772610

Wistreich A. G., (1997). Microbiology laboratory, Fundamentals and Applications. East Los Angeles College. Prentice Hall, Upper Saddle River, New Jersey 07458.

APPENDICES

Appendix 1

Questionnaire for Market Survey

Shop No.-

Ward No.-

1. What are the different types of preserved fish available in your shop?

- Smoked
- Salted
- Sun-dried
- Others

2. What are the varieties in preserved fishes?

Smoked	Salted	Sun-dried

3. What are the sources of supply?

- Nepal
- India
- Bangladesh
- Others

4. If the fishes are from Nepal then from which part of Nepal they are brought?

5. What are the prices (per kg) of different varieties of preserved fish?

6. What are the different purposes of these fish?

7. What is the best selling season of fish?

8. What do you do with the unsold fish?

a) I will try to sell in cheaper price ( )

b) I sell them to feed producers ( )

c) I eat them myself ( )

d) I them to beggars free of cost ( )

e) I dispose them

f) Others specify ( )

9. What are the measures taken to keep these fishes free from bacterial and fungal contamination?

10. What do you think you can do to improve the hygienic condition of the shop?

APPENDIX 2

Composition of Media Used in Isolation and Identification of Bacteria and Fungi

Culture Media

1. Nutrient Agar (NA)

Composition	**(gram/litre)**
Peptone	5.0
Sodium Chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0
Final pH (at 25^0C)	7.4±0.2

2. Nutrient Broth (NB)

Composition	**(gram/litre)**
Peptone	5.0
Sodium Chloride	5.0
Beef extract	5
Yeast extract	1.5

Final pH (at 25^0C)	7.4±0.2

Sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes.

3. Potato Dextrose Agar (PDA)

Composition	**(gm\litre)**
Potato Infusions	200
(infusion from 200g potatoes)	
Dextrose	20
Agar	15.00

Final pH (at 25^0C)	5.6±0.2

The low pH value partially inhibits the growth of the accompanying bacterial Growth.

Composition of Stains and Reagents

1 Gram's staining

Stains

i) Crystal violet

) **Solution A**

Crystal Violet	2.0gm
95% ethyl alcohol	20.0ml

- **Solution B**

Ammonium oxalate	0.8gm
Distilled water	30.0ml

Crystal violet was dissolved in ethyl alcohol and ammonium oxalate in distilled water. Then solution A and B are mixed.

i) Gram's Iodine

Iodine	1.0gm
Potassium iodide	2.0ml
Distilled water	300.0ml

Iodine and potassium iodide were dissolved in distilled water.

ii) Ethyl Alcohol (95%)

Absolute alcohol	95.0ml
Distilled water	5.0ml

iii) Safranin

Safranin	10.0ml

(2.5 % solution in 95% ethyl alcohol)

Distilled water	100.0ml

Catalase Test

Reagents: (3 % Hydrogen peroxides).

Composition	**(gm\Litre)**
Concentration hydrogen peroxide	3ml
Distilled water	97ml

Oxidase Test

Oxidase test is done to determine the presence of the oxidase enzyme. Oxidase reaction is due to the presence of a cytochrome oxidase system.

Oxidase reagent

Whatman No. 1 filter paper was cut into strips of 6-8 cm in diameter. It was soaked in the reagent till saturation. The paper strips were drained and freeze dried and stored in a dark tightly **sealed bottle.**

Procedure

The Oxidase test paper was moistened with distilled water. A colony was picked using glass rod and rubbed to the paper. Development of violet colour within 10 seconds is an indicative of positive test.

Sulfide-Indole-Motility Medium (SIM)

Sulfide-Indole-Motility is a semi solid medium used for the determination of sulfide production, Indole formation and motility of enteric bacteria.

Composition	**(gm/litre)**
Beef extract	3.0
Peptone	30.0
Peptonized iron	0.2
Sodium thiosulfate	0.025
Agar	3.0

Final pH (at 25^0C)	7.3±0.2

Indole

Composition	**(gm/litre)**
Peptone containing tryptophan	10.0gm
Sodium Chloride	5.0gm
Distilled water	1000.0ml

Reagent: Kovac's reagent

Composition	**(gm/litre)**
P-dimethyl aminobenzaldehyde	5.0gm
Isoamyl alcohol	75.0ml
Concentrated hydrochloric acid	25.0ml

MR-VP medium (glucose-phosphate broth).

Composition	**(gm/litre)**
Buffered peptone	7.0
Dextrose	5.0
Tripotassim phosphate	5.0

Final pH (at 25^0C)	6.9±0.2

Reagent – Methyl Red

Composition	**(gm/litre)**
Methyl red	0.04gm
Ethyl alcohol	40.0ml
Distilled water	60.0ml

Voges – proskauer

Medium -MR-VP medium (Glucose – phosphate broth)

- **Solution A**

ά-naphthol	5.0gm
Ethyl alcohol (95%)	100.0ml

- **Solution B**

Potassium hydroxide	40.0gm
Distilled water	100.0ml

Citrate

Medium – Simmon's Citrate Agar

Composition	**(Gram/Litre)**

Monoammonium phosphate	1.0
Dipotassium phosphate	1.0
Sodium Chloride	5.0
Sodium Citrate	2.0
Magnesium Sulphate	0.2
Bromothymol blue	0.08
Agar	15.0
Final pH (at 25^0C)	6.8±0.2

Hydrogen Sulfate Test (Triple Sugar Iron Agar Test)

Composition	**(gm/litre)**
Peptone	10.0
Tryptone	10.0
Yeast extract	3.0
Beef extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous sulfate	0.2
Sodium Chloride	5.0
Sodium Thiosulfate	0.3

Phenol- red	0.024
Agar	12
Final pH (at 25^0C)	7.4±0.2

Urease

Medium – Urea agar base.

Composition	**(gm/Litre)**
Peptone	1.0
Dextrose	1.0
Sodium Chloride	5.0
Disodium phosphate	1.2
Monopotassium phosphate	0.8
Phenol red	0.012
Agar	15.0
Final pH (at 25^0C)	6.8±0.2.

Hugh and Leifson Medium

Composition	**(gram/Litre)**
Peptone	20.0
Sodium Chloride	50.0
Dipotassium Phosphate	3.0

Agar	20.0
Bromo Thymol Blue	0.5
Glucose	100.0
Final pH	7.1±0.2

All the above mentioned media belonged to **HiMedia Laboratories Limited, Mumbai-400 086, India**

Appendix 3

List of Materials used:

Materials	**Company Name**
Autoclave	
Oven	(NSW India)
Incubator	(NSW) India
Microscope	Olympus
Refrigerator	Samsung
Laminar air flow	(EACI)
Glass wares	Pyrex and Borosil
Test tube stands	
Weighing Balance	(Fischer Scertfi)
Media	HiMedia Laboratories Limited