MATERIALS AND METHODS
Sample

There are several species of carps available. Only *Cyprinus carpio* var. *communis* (Linn.) was selected for the present investigation considering its commercial importance and availability.

**Systematic position of *Cyprinus carpio***

<table>
<thead>
<tr>
<th>Category</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum</td>
<td>Chordata</td>
</tr>
<tr>
<td>Class</td>
<td>Pisces</td>
</tr>
<tr>
<td>Order</td>
<td>Cypriniformes</td>
</tr>
<tr>
<td>Super order</td>
<td>Cyprinoidei</td>
</tr>
<tr>
<td>Family</td>
<td>Cyprinidae</td>
</tr>
<tr>
<td>Subfamily</td>
<td>Cyprinae</td>
</tr>
<tr>
<td>Genus and species</td>
<td><em>Cyprinus carpio</em> var. <em>communis</em> (Linnaeus)</td>
</tr>
</tbody>
</table>

**Characteristics of the species**

A close view of the specimen is shown in Plate-1

<table>
<thead>
<tr>
<th>Common name</th>
<th>scale carp</th>
</tr>
</thead>
<tbody>
<tr>
<td>English name</td>
<td>common carp</td>
</tr>
<tr>
<td>Scientific name</td>
<td><em>Cyprinus carpio</em> var. <em>communis</em> (Linn.)</td>
</tr>
</tbody>
</table>
1. The marketing sized Cyprinus carpio var. communis (Linn.) immediately after catch (alive).

2. Common carp immediately after death.

3. Spoiled common carp - note the engorged belly.
Distinguishing characters

D iii-iv 18-20; A iii 5; P i 15; Vi 18. Body stout, slightly compressed, head moderate, triangular; snout obtusely rounded. Mouth is small, oblique and protrusible with thick and fleshy lips. There are two pairs of barbels where maxillary barbels twice as long as rostral pair. Gill rakers 21-29 on first arch. Dorsal fin inserted midway between snout-tip and base of caudal fin; dorsal spine stout, serrated behind. Anal fin trapezoidal; pectoral fins large and rounded; caudal fin deeply emarginated. Scales large and lateral line straight with 30-40 scales.

Colour in life, rather variable, adaptable to various situations and the character of the bottom of the ponds where it lives, usually olivaceous, with silvery or golden sides. Fins yellowish, reddish or golden; anal fins becomes bright red in breeding season.

Geographical distribution

The original occurrence of common carp C. carpio was restricted to a narrow belt in Central Asia within latitudes 35-135°E and altitude generally 300m above Mean Sea Level (Jhingran, 1982). It is the native of Europe but most widely cultivated species all over the world. Cyprinus carpio var. communis (Linn.) (Bangkok strain), a Chinese carp was introduced in India in 1939 and 1957 from Sri Lanka and Thailand. It is distributed through out the tropical, sub-tropical and temperate regions. The carps and a few other members of the taxonomic family Cyprinidae are the most commonly cultured freshwater fish species in China, Taiwan, India, Bangladesh, Indonesia, Malaysia, Nepal, and Vietnam owing to their economic importance (New et al. 1995).
Food and feeding

The common carp feeds at the surface, on the bottom, or in midwater on plants, algae, snails, worms, and insect larvae, shrimps, murrels and many other aquatic organisms. It opens its mouth wide and sucks its food in like a vacuum cleaner and often rummages through the bottom detritus, sending up clouds of silt and uprooting plants.

Growth

In India, this carp attains maturity within 5-6 months and breeds naturally in the pond (Jhingran, 1982). It weighs around 1 kg in the first year and reaches the maximum weight of 6 kg. It grows faster and accounts for nearly 46% of the total yield in polyculture (Anon., 1977).

Breeding

*C. carpio* spawn in late spring and early summer, when the water temperature exceeds 18°C. The eggs are laid in shallow water and they hatch in five to eight days, the hatchings initially remaining attached to the plants. The young fish grow very quickly.

Commercial significance

The common carp is the most valuable freshwater fish, which is readily appreciated by anglers. It occupies third place in aquaculture production. It’s total yield in 1992 was over 1,200,435 metric tonnes (FAO, 1992). *Cyprinus carpio* production in 1995 was 1,803,256 metric tonnes. India's production of common carp alone was 13705 metric tonnes in 1996 (FAO, 1998). The major parts of carps are consumed locally in the producing countries mainly China and India. Common carp can be reared
in polyculture systems that make optimum use of the natural productivity of ponds and water bodies where they are stocked. They have good markets in Asian countries due to traditions and relatively low prices.

Collection of samples

For the present study, the edible freshwater fish *Cyprinus carpio* with the length of 30-35 cm and weighing 500-600 g were collected from the fish culture pond of Government Fisheries Department, Manimuthar, Tamilnadu State, India. The fish were brought to the laboratory using sterile plastic buckets containing pond water where the fish live. The fish samples were brought to the laboratory within two hours after collecting the samples for microbiological and biochemical analyses.

Treatment

On arrival to the laboratory, the fish were killed by shock treatment. The fish samples were divided into three lots as shown below.

i) Raw whole fish were taken in sterile plastic trays and stored at ambient temperature (28 ± 2°C) and another set was stored in the refrigerator (4 ± 1°C). Samples were evaluated for sensory, biochemical and microbiological attributes at 0, 2, 4, 6, 8, 12 and 24 hours (ambient temperature) and at 0, 1, 2, 3, 6, 12 and 21 days (refrigeration temperature).

ii) The second lot was processed as fillets, packed and stored at ambient and refrigeration temperatures. As described for raw fish
samples, these fish fillets were also processed for sensory, biochemical and microbiological attributes.

iii) The third lot was also filleted, processed and preserved with chemical preservatives and stored in the refrigerator. These fish fillets were also processed for sensory, biochemical and microbiological analyses.

Strict aseptic procedures were adopted throughout the experiments in order to avoid innocuous entry of laboratory contaminants. Triplicates and controls were maintained throughout the period of this study.

Preparation of Fish Fillets

On arrival to the laboratory the fish were beheaded, scaled and gutted manually. The dressed fish were then washed with tap water thoroughly to remove adhering dirt and slimly particles. The fillets were again washed, drained well and wrapped in gamma irradiated polythene bag. The stored fillets were examined and evaluated for its organoleptic, biochemical and microbiological qualities at intervals of 0, 2, 4, 6, 8, 12 and 24 hours under ambient temperature and at intervals of three days upto three weeks under refrigeration. As hastier deterioration of fish samples were observed, shorter sampling schedules were employed in the case of fillets stored in ambient temperature. On the other hand, in view of the retarded rate of deterioration of fillets stored at 4°C, samples were drawn at three days intervals so as to obtain distinct results.
WORK PLAN

Fresh Fish - common carp

Washed with tap water

Divided into two lots

Stored as Whole fish

At room temperature

28 ± 2°C

Sampling at regular intervals

Hours

Days

0 2 4 6 8 12 24

0 1 2 3 6 12 21

Stored as Fillets

Beheaded, gutted and scaled
Washed to remove blood

Filleted; Divided into two lots

Fillets packed in polythene bags

Fillets dipped in 2% w/v preservative solutions

Sealed untreated control

Sodium chloride

Sodium acetate

Potassium sorbate

Sodium acetate and potassium sorbate in equal parts

Dipped for 30 minutes (fish fillet : treatment solution 1 : 2 w/v)

Drained

Packed in polythene bags and sealed

Stored in refrigerator (4°C)

Sampling at 3 days interval upto 21 days
PLATE - II

FILLETS

Photographs showing the dorsal evicerated and inner surfaces of fillets stored in a refrigerator.

1

2
Method of preservation of Fish Fillets

Groups of five pairs of fillets in triplicates were arranged and dipped in the treatment solutions. Preservative solutions of guaranteed grade crystalline Sodium chloride, anhydrous powder of Sodium acetate (Merck, Mumbai), granular food grade Potassium sorbate (Chemco Fine Chemicals, Mumbai) and the combinations of Sodium acetate and Potassium sorbate were prepared in glass distilled water at 2% w/v concentrations. Fillets were divided into five lots packed in gamma irradiated polythene bags with or without preservatives as per the details described below.

The untreated fillets (one pair) were packed in a polythene bag to serve as control. Remaining four pairs of fillets were dip treated in respective preservative solutions for 30 minutes (fish fillet: treatment solution in the ratio 1:2 w/v). Using flame sterilized and stainless-steel tongs, dipped fillets were taken out after 30 minutes and placed on a clean stainless steel grill inorder to drain the excess preservative.

Packaging and storing

Then the fillets were packed in prelabelled sterile polythene bags (two fillets in each bag) using semi automatic hermetic seals. The sealed packs were arranged in plastic containers and stored in a refrigerator under aseptic condition. Temperature of the refrigerator was monitored regularly to achieve a constant temperature of 4°C.

Sampling and analysis of Fish Fillets

Sampling was performed for microbiological and biochemical
Preservatives treated fillets before storing in the refrigerator.

C - Control
T₁ - Sodium chloride
T₂ - Sodium acetate
T₃ - Potassium sorbate
T₄ - Sodium acetate and potassium sorbate
analyses on the day after packaging (initial day). Thereafter the samples were taken at three days interval for three weeks. One fillet from each treatment was taken for organoleptic, biochemical and microbiological analysis. Each package was opened gently and sterile tongs were used to take out the fillets. All the analyses for spoilage studies were done simultaneously in triplicate.

**Estimation of spoilage**

**A. Organoleptic Assessment**

Organoleptic assessment (sensory evaluation) of the whole fish was performed by a panel of five persons (Research scholars of the Environmental Biotechnology Department, Sri Paramakalyani centre for Environmental Sciences, Alwarkurichi, Tirunelveli District, Tamilnadu) while making observations emphasis was laid on general appearance, slime formation on the body surface, nature of eyes, colour of gills, mucous and skin, texture, odour and overall acceptability. Five-point scale suggested by Brenner (1985) was modified and used for assessing the sensory characteristics of the fillets (Appendices I to V). Score awarded for each attribute were pooled and the mean panel scores were calculated. Using 5-point hedonic scale 5, 0 and 3 were being considered as very good, bad and just unacceptable respectively.

**B. Biochemical Analysis**

To assess the biochemical changes in the fish samples during storage and spoilage various analyses were carried out by maintaining triplicates and control.
Panel of judges assessing the quality of fish fillets by means of appearance, texture and odour.
Estimation of moisture

Moisture content of fish samples was estimated adopting the method of AOAC (1995) and the results were expressed in percentage. Ten grams of well ground sample was taken in a preweighed porcelain dish and spread uniformly. Then the dish was heated using a hot air oven at 100 ± 2°C for about sixteen hours. After heating, the dish was cooled in a desiccator and weighed. Repeated the process of heating, cooling and weighing until the difference in weight between two successive readings was less than 1 mg. Recorded the lowest weight and calculated the moisture percentage, using the following formula.

\[
\text{Moisture content (\%) = } \left[ \frac{(W_1 - W_2)}{(W_1 - W)} \right] \times 100
\]

Where, 

- \( W \) - Weight of the empty dish.
- \( W_1 \) - Weight of the dish with sample before drying.
- \( W_2 \) - Weight of the dish with sample after drying.

pH (Hydrogen ion concentration)

Approximately 5g of fish meat were blended well in a teflon tissue homogenizer with 50 ml distilled water and the pH of the homogenate was measured using pH meter (Systronics digital pH meter 335).

Protein

Protein content of the fish was estimated by adopting the method of Lowry et al. (1951) using Bovine Serum Albumin (BSA) as standard, five gram of the sample was weighed and homogenized in a teflon homogeniser with 5 ml distilled water. After centrifugation at
12,000 rpm for 30 minutes in a refrigerated centrifuge the supernatant was estimated for the protein content and BSA standard graph was prepared. To prepare protein calibration graph, BSA stock solution of 50 mg / 50 ml was made in glass distilled water. From this 0.2, 0.4, 0.6 ... 1.0 ml was pipetted out and made up to 1.0 ml with glass distilled water in a boiling tube. Distilled water blank without BSA was also maintained. To all these tubes, 5 ml of alkaline mix was added and allowed to stand for 10 minutes. This was followed by the addition of 0.5 ml of Folin-Ciocalteau reagent, mixed well and allowed to stand at room temperature in dark for 30 minutes. The absorption of colour developed was measured at 660 nm in a spectrophotometer (Spectronic-20). One ml of the supernatant was processed as per the methodology adopted for BSA standards and the optical density ($OD_{660}$) was recorded and the amount of protein present in the sample was calculated using standard graph.

**Lipid**

Quantitative estimation of lipid of different samples of *C. carpio* was done according to the gravimetric method of Folch *et al.* (1956). Two grams of the fish sample was ground in a tissue homogenizer with 5 ml of chloroform-methanol mixture (2:1 v/v) and was allowed to stand for 30 minutes at room temperature. The homogenate was centrifuged at 13,000 rpm for 15 minutes at room temperature and the supernatant was transferred to a fresh stryfoam tube. This process of homogenisation and decanting the supernatant into the centrifuge tube was repeated for three times. The supernatant was then transferred to a glass weighing bottle of known weight and was dried in an oven maintained at 50°C.
After complete drying the containers with the fat were weighed, checked for constant weight and calculations were made, using the following formula:

\[
\text{Lipid percentage of the given sample} = \frac{(X - Y)}{W} \times 100
\]

Where,  
- \( X \) - lowest weight of the container with fat  
- \( Y \) - weight of the container  
- \( W \) - weight of the sample

**Carbohydrate**

Carbohydrate content of fish samples were estimated following Phenol-Sulphuric acid method of Dubois et al. (1956). Tissue homogenate was prepared by mixing fresh sample and distilled water in a tissue grinder in the ratio 1.4 (w/v) and made upto 50 ml with distilled water. It was centrifuged at 12,000 rpm for 30 minutes in a refrigerated centrifuge. From this one ml of the supernatant was used for glucose estimation. To prepare glucose calibration graph, D-glucose standard solution of 10 mg / 100 ml was made in glass distilled water. From this, 0.2, 0.4, 0.6, 0.8 and 1.0 ml was pipetted out and made upto 1.0 ml with glass distilled water in a series of test tubes. Distilled water blank without glucose was also maintained. One ml of 5% phenol solution and 5ml of conc. sulphuric acid were added in each tube. The solution was mixed thoroughly with a glass rod and allowed to cool to room temperature for 30 minutes. Optical density of the solution was then measured at 490nm and the unknown values were calculated using the standard graph. The amount of carbohydrate present in the fish samples were expressed in percentage.
Ammonia

In order to estimate the ammonia content of fish samples, tissue homogenate was prepared by mixing muscle tissue of fish fillets and distilled water in a Teflon tissue grinder in the proportion of 1 : 4 (w/v) and made up to 5 ml with glass distilled water. It was centrifuged at 12,000 rpm for 30 minutes in a refrigerated centrifuge. From the supernatant one ml was drawn, nesslerized directly (APHA, 1976) and optical density was measured at 410nm. Standard curve was prepared with ammonium sulphate 94.3 mg dissolved in 100 ml distilled water and calculations were made accordingly.

Peroxide value (PV)

Peroxide value of fish samples were estimated adopting the method of AOAC (1975) and the results were expressed as meq/kg fat. Minced fish sample (5-10g) was weighed and ground in a surface sterilized mortar and pestle. The fish sample was ground well using anhydrous sodium sulphate until all the water got removed. Then an extract was made with 30 ml of chloroform and the contents were filtered. 10 ml of glacial acetic acid was added into a conical flask containing the filtrate. To the above solution, 10 ml of 10% potassium iodide was added, stoppered and kept in a dark place for 10 minutes with occasional shaking. Then 50 ml of distilled water and 1 ml of starch solution were added and the liberated iodine was titrated against 0.01 N standard sodium thiosulphate solution. The peroxide value was calculated as ml of 0.01 N sodium thiosulphate solution for 1kg of fat using the following formula.
Peroxide value = \[(ml \times N) / g\] \times 1000 milli equivalent of peroxide / kg of fat.

**Free Fatty Acids (FFA)**

Free Fatty Acid of different samples of fish were estimated using the titrimetric method of AOAC (1975) and the results of FFA were expressed as percentage of oleic acid. Minced fish sample (5g) was weighed accurately and ground in a surface sterilized mortar and pestle and add 20ml of the solvent mixture of chloroform, methanol, and isopropanol in the ratio of 2:1:2. Then this content was titrated against 0.05 N standard potassium hydroxide solution using three drops of 0.05% metacresol purple as indicator. The end point was the appearance of a purple colour. Amount of potassium hydroxide needed for neutralizing the free fatty acid content of the sample was noted. The results were expressed as free fatty acids as percentage of oleic acid per gram of fat, using the following formula

\[
\text{Free Fatty Acid} = \frac{(V \times 0.028)}{(W \times 2)} \text{ oleic acid} / \text{g of fat.}
\]

where,  
- \( V \) - Volume of Potassium hydroxide used.  
- \( N \) - Normality of Potassium hydroxide used.  
- \( W \) - Weight of the sample used.

**Total Volatile Base Nitrogen (TVB-N)**

Total volatile bases and trimethylamine contents of fish samples were estimated employing microdiffusion method of Conway (Conway, 1950) using trichloroacetic acid extract of the muscle. Results were expressed in milligram percentage. Accurately 10g of the sample was
weighed and homogenized with 10ml of distilled water. 10ml of 5% trichloroacetic acid was added, mixed well and centrifuged at 12,000 rpm for 30 minutes in a refrigerated centrifuge. The supernatant was made upto 50 ml in a volumetric flask using distilled water. 1 ml of the sample was pipetted out into the outer chamber of Conway unit. Then 1ml of N/70 hydrochloric acid was added to the sample and also a drop of mixed indicator (dissolved 80 mg of bromocresol green and 16 mg of methyl red in 100 ml of 95% ethanol) were added into the inner chamber. Then 1 ml of saturated potassium carbonate solution was poured into the outer chamber. The unit was covered with a ground glass plate smeared with vacuum grease to give airtight contact. The outer chamber contents were mixed gently and allowed to stand undisturbed overnight. The excess acid in the inner chamber was titrated against standard sodium hydroxide (N/70) taken in a burette and dipping its tip in the inner chamber. A blank was carried out with 1 ml of distilled water in the outer chamber. Amount of acid used by the bases released from the fish sample was calculated using the following formula and referred as total volatile bases.

\[ 1\text{ml of N}/70\text{ HCl} = 0.2\text{ mg of N}. \]

**Tri methylamine Nitrogen (TMA-N)**

TMA-N content was also determined adopting microdiffusion method of Conway (Conway, 1950) adding formaldehyde. 0.5 ml of formaldehyde was added into the outer chamber just before the addition of saturated potassium carbonate solution. TMA-N content of the fish samples were calculated as described for Total volatile base nitrogen.
Alpha amino nitrogen (AAN)

Alpha amino nitrogen was determined employing the method of Pope and Stevens (1939) using trichloroacetic acid extracts. 10g of fish muscle with 20 ml of trichloroacetic acid were taken. The volume was made up to 100 ml with distilled water mixed well and filtered. 15 ml of this extract was measured into a 50 ml graduated glass cylinder. To this four drops of thymolphthalein and sufficient amount of 1N sodium hydroxide was added to produce a light blue colour. Then 20ml of copper phosphate suspension was added and the volume was made to 50ml using distilled water. The contents were mixed well and filtered through a Whatman No. 1 filter paper. To 10ml aliquots of the filtrate from the 50ml of sample, 0.5ml of glacial acetic acid and 1g of potassium iodide were added. The contents were titrated against 0.01N sodium thiosulphate at a rapid rate until the solution becomes pale yellow. Then 1.2ml of starch solution was added and the titrations were repeated. The end point was the disappearance of blue colour. The amount of alpha amino nitrogen in the given sample was calculated from the titre value.

C. Microbiological Analysis

Total heterotrophic, psychrophilic and hydrogen sulphide producing bacteria, bacterial generic composition and their physiological characteristics were determined as per the procedure specified in AOAC (1995).

Enumeration of Total Heterotrophic Bacterial (THB) Population

The THB population in muscle, gill and gut of raw whole fish and in muscle of the common carp, Cyprinus carpio fillets were enumerated.
Preparation of samples

Muscle tissue

The surface of the fish C. carpio was rinsed thoroughly with sterile distilled water to remove the adhering dust particles. A portion of the muscle tissue of the fish (10g) was weighed in a surface sterilized (with alcohol) monopan balance aseptically and homogenized well with 10ml of sterile saline (0.85% NaCl) using surface sterilized mortar and pestle. From this homogenate, 10ml was transferred to 90ml of sterile saline (0.85% NaCl) blank in a Erlenmeyer flask (10\textsuperscript{1} dilution). From this, 1ml aliquotes were withdrawn aseptically using micro pipettes and decimally diluted with 9ml sterile saline blanks and used as inoculum for enumeration of total heterotrophic bacterial counts (THB).

Gill

Gill rackers of the fish C. carpio was removed aseptically, weighed (1g) and homogenized with 10ml of sterile saline. This was then serially diluted using 9ml saline blank and used as inoculum for enumeration.

Gut

The fish C. carpio was dissected and the gut was removed aseptically. One gram of gut tissue was processed as per the methodology adopted for muscle tissue and serially diluted samples were used as the inoculum for enumeration.

Preparation of serial dilutions and plating procedures

Saline suspensions (10\textsuperscript{1} dilution) of fish sample homogenates (muscle tissue, gill and gut) were further serially diluted separately upto 10\textsuperscript{7} dilutions with 9ml of sterile saline blank. From each dilution, one ml
 aliquote was aseptically withdrawn using a sterile glass pipette and transferred as inoculum into sterile petriplates. In this, autoclaved (15 lbs. inch² for 15 minutes) and cooled (45°C) nutrient agar medium (Himedia, Mumbai) was poured to the maximum quantity of 15-20 ml, swirled gently to ensure complete mixing of inoculum with the medium and allowed to solidify. These plates were inverted and incubated at 37°C for 24-48 hours. Petriplates with discrete colonies of THB of countable range (30-300) were enumerated and expressed as cfu/g of sample.

**Composition of the Nutrient agar**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5g</td>
</tr>
<tr>
<td>Beef / yeast extract</td>
<td>3g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>3g</td>
</tr>
<tr>
<td>Agar</td>
<td>20g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.2 ± 0.2</td>
</tr>
</tbody>
</table>

**Enumeration of psychrophilic bacteria**

The psychrophilic bacterial population of different samples of the fish *C. carpio* were enumerated as described for THB except that the incubation temperature which was maintained at 3-7°C for seven to ten days (Cousin *et al.* 1992).

**Enumeration of hydrogen sulphide producing (sulfidogenic) bacteria**

Hydrogen sulphide producing bacteria (sulfidogenic) were enumerated adopting methodology employed in the enumeration of THB.
The major deviation from this procedure to enumerate sulfidogenic bacteria is that, Peptone Iron Agar (PIA) was used (Levin, 1968) instead of nutrient agar medium. Occurrence of H₂S producing bacteria was recorded on the basis of appearance of black colonies in this agar plates. Plates with countable range of black colonies (30-300) were enumerated and expressed as colony forming units per gram weight of the fish samples (cfu/g).

**Isolation and maintenance of bacterial cultures**

Isolations were made at random from nutrient agar medium plates containing countable number of colonies (30-300 colonies per plate). After recording the morphological characteristics of the colony such as opacity and pigmentation, they were purified by quadrant streaking and stored in nutrient agar slants. The isolates were checked for their purity by repeated streaking on nutrient agar and were maintained on nutrient agar slants in the laboratory in a refrigerator at 4°C to keep the bacterial strains viable.

**Identification of bacterial isolates**

Around 40 colonies were selected for each sample, as it was sufficient to obtain a representative diversity of bacterial communities (Bianchi and Bianchi, 1982). The isolates were characterized up to genus level on the basis of gram stain, spore stain, motility, oxidase, catalase and O/F tests and grouped into various genera as per the scheme recommended by Surendran and Gopakumar (1981a) and Bergey's manual of determinative Bacteriology (Buchanan and Gibbons, 1974).
SCHEME FOR IDENTIFICATION OF ISOLATES
(Surendran and Gopakumar, 1981a)
Gram staining (16-24 hours culture)

- Gram positive
  - Cocci
    - (Micrococcus sp.)
  - Rod
    - (Lactobacillus sp.)
      - Catalase +ve
      - Nospore
      - (Arthrobacter sp., Corynebacterium sp.)

- Gram negative
  - Penicillin sensitivity 2.5 l.U / disc.
    - +ve
    - -ve
      - Pigmentation
        - +ve
          - White
            - (Achromobacter sp.)
            - Yellow
              - (Flavobacterium sp.)
        - -ve
          - Green
            - (Pseudomonas sp.)
      - Fermentation
        - +ve
          - Yellow
        - -ve
          - Acid and gas
            - (Moraxella sp.)
            - (Alcaligenes sp.)
          - Acid / no gas
            - (Vibrio sp.)
  - Kovac's oxidase test
    - +ve
    - -ve
      - Luminescent
        - (Photobacterium sp.)
      - Non-luminescent black
        - (Aeromonas sp.)
      - Fermentative
        - (Enterobacter sp.)
      - Non-fermentative
        - (Acinetobacter sp.)
Production of hydrolytic enzymes by bacterial isolates

The ability of the bacterial isolates drawn from fish samples to elaborate various hydrolytic enzymes such as gelatinase, caseinase, lipase and amylase was determined by plate assay.

Gelatinase

Gelatinase producing ability of various bacterial isolates from the fresh and spoiled fish was tested by employing modified Frazier’s gelatin agar medium of Harrigan and McCance (1972) of the following composition.

Composition of the Gelatin agar

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Meat extract</td>
<td>10 g</td>
</tr>
<tr>
<td>Gelatin</td>
<td>4 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>15 g</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.2 ± 0.2</td>
</tr>
</tbody>
</table>

The prepared medium was autoclaved, poured into sterile petriplates and allowed to solidify and air dried overnight. Nutrient broth cultures of bacterial isolates were inoculated by surface streaking on the solidified agar medium and incubated at 37°C for 48 hours. The plates were flooded with 8-10 ml of the test reagent (mercuric chloride, 15g; conc. hydrochloric acid, 20 ml and distilled water, 100 ml). Gelatin hydrolysis was identified by the appearance of clear halos around the colonies.
Caseinase

Caseinase production by different cultures was detected by employing casein agar medium of Harrigan and McCance (1972) of the following composition.

**Composition of the Casein agar**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Meat extract</td>
<td>10 g</td>
</tr>
<tr>
<td>Casein (BDH)</td>
<td>30 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>15 g</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.2 ± 0.2</td>
</tr>
</tbody>
</table>

Bacterial isolates were inoculated on the casein agar plates by surface streaking. The inoculated plates were incubated at 37°C for 48 hours. Caseinase enzyme production was detected by the appearance of clear zones around the colonies.

Lipase

Production of lipase by different bacterial strains were tested using Tween-80 agar medium (Harrigan and McCance 1972) of the following composition.

**Composition of the Tween agar**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>100 g</td>
</tr>
</tbody>
</table>
Tween - 80 : 10 ml
(sorbitol monooleate)
Sodium chloride : 15 g
Agar : 20g
Distilled water : 1000 ml
pH : 7.2 ± 0.2

The bacterial strains were made single streak inoculation on the Tween 80 agar plates and the inoculated plates were incubated at 37°C for 48 hours. Lipase production was detected by the appearance of opaque zones around the colonies. Appearance of a waxy material around the colonies was the indication of the liberation of insoluble oleic acid formed as a result of lipase action.

**Amylase**

Amylase production of different bacterial strains were tested on the starch agar medium (Harrigan and McCance 1972) of the following composition.

**Composition of the Starch agar**

- Peptone : 10 g
- Meat extract : 100g
- Starch (soluble) : 2g
- Sodium chloride : 15g
- Agar : 20g
- Distilled water : 1000 ml
- pH : 7.2 ± 0.2
Photographs showing the production of hydrolytic enzymes by bacteria isolated from the fillets of *C. carpio* during spoilage.

1. C - Caseinolytic
2. G - Gelatinolytic
3. L - Lipolytic
4. A - Amylolytic
The bacterial strains were inoculated and incubated using starch agar plates as described for lipase production. The production of amylase was tested by flooding the plates with Gram's iodine solution (potassium iodide, 2g; iodine, 1g and distilled water, 300 ml). Unhydrolysed starch formed a blue colour with iodine. The amylolytic colonies developed clear zones around them.

**Hydrogen sulphide production test:**

Sulfidogenic bacterial strains isolated from fish samples that were enumerated on PIA were further cross checked for their hydrogen sulphide production characteristic. This was carried out by surface streaking the nutrient broth culture of these isolates on air dried Triple Sugar Iron (TSI) agar slants of the following composition and incubated at room temperature (28°C) for 48 hours. Production of hydrogen sulphide was recognized as on the basis of black butts and raised slant.

**Composition of the TSI agar**

- Yeast extract : 3g
- Beef extract : 3g
- Peptone : 15g
- Protease peptone : 5g
- Lactose : 10g
- Sucrose : 10g
- Glucose : 10g
- Ferrous sulphate : 0.2g
- Sodium chloride : 0.48g
D. Statistical Analysis

All the experimental data were tested by two way ANOVA to understand the interactions of various storage conditions, preservatives on sensory, biochemical and microbiological parameters according to the methods described in Snedecor and Cochran (1967). Coefficient of correlation was used to find out the relationship between various parameters and storage period, and to test the significant differences of all the variables analysed in the present investigation.

The variables include spoilage indices like PV, TMA-N, TVB-N, NH₃, AAN, FFA, THB, H₂S producing and psychrophilic bacteria; proximate components such as protein, carbohydrate, lipid, moisture and pH; spoilage flora-Vibrio sp., Pseudomonas sp., Aeromonas sp. and Micrococcus sp., and hydrolytic enzyme producing bacteria-gelatinolytic, caseinolytic, lipolytic and amylolytic.