Materials and Methods

3.1. Collection of diseased fish

Infected fishes *Catla catla, Labio rohita, Cirrhinus mrigala, Channa punctatus, C. gachua, Clarias batrachus, Heteropneustes fossilis, Puntius* sp., *Mystus* sp. were collected from affected ponds of Ambari, Fulbari, Gajaldoba of Jalpaiguri district and Chathat, Leusipakuri of Darjeeling district. Infected fish were also collected from local markets. The fish were brought to the laboratory in live condition and were kept in glass aquaria measuring 90 x 35 x 35 cm in which the depth of water was 20 cm.Half of the static water was changed every day.

3.2. Collection and maintenance of healthy fish

Healthy *Channa punctatus* were collected from ponds of Sonapur of Darjeeling district with no record of EUS outbreak in various quantities time to time for the experimental work.

The healthy fish were kept kin glass aquaria measuring 90 x 35 x 35 cm in which depth of water was 20 cm. The fishes were fed regularly with chopped earthworms. All fishes were acclimatized under laboratory conditions for at least 15 days before using them for experimental work.

Healthy carp, *Cyprinus carpio* (30 - 50 g) were collected from local sources and acclimatized in glass aquaria measuring 90 x 35 x 35 cm in which depth of water was 20 - 25 cm at a density of 5 fishes per aquarium tank with constant blowing of air through water by air pump in laboratory. The water temperature varied from 28 to 30^{0}C. The fishes were fed Tokyu fish food.

3.3. Fungal culture

3.3.1. Source of culture

Fungi were isolated from infected fishes, during the course of the work. Culture were maintained aseptically by transferring the fungus to freshly prepared fungal media. Code names were given to each of the isolates. Fungal isolate (F_{cs1}) was used for experimental work.

3.3.2. Composition of fungal media

Various types of culture media were used during this study. The names and composition of those media are given below.

GP (Glucose-peptone) Broth (Lilley *et al.*, 1998)

Glucose	3 gm
Peptone	1 gm
$MgSo_4.7H_2O$	0.128 gm
KH_2PO_4	0.014 gm
$CaCl_2.2H_2O$	0.029 gm
$Fecl_3.6H_2O$	2.4 mg
$MnCl_2.4H_2O$	1.8 mg
$CuSO_4.5H_2O$	0.4 mg
Distilled water	1000 mL

All the ingredients were dissolved in distilled water and then autoclaved at 121^0C for 15 minutes.

GP (Glucose-peptone) Agar: 12 mg/L of agar powder was added to GP medium to prepare GP agar.

GPY (Glucose-peptone-yeast extract) Broth: 0.5 gm/L of yeast extract was added to GP medium to prepare GPY medium.

GPY (Glucose-peptone-yeast extract) Agar: 0.5 gm/L of yeast extract and 12 gm/L of agar powder were added to GP medium to prepared GPYA medium.

Penicillin-K, 100 units/mL and oxonilic acid were added at concentration of 100 mg/L to the media after autoclaving and cooling to 50^0C. (Roberts *et al.*, 1993).

Autoclaved pond water: The pond water is known to promote the fungal growth. Pond water was first filtered through Whatman filter proper. One part

of the filtered pond water was mixed with two part of distilled water and then autoclaved.

Material for fungal stain

1% Lactophenol cotton blue

Cotton blue powder	1 gm
Dissolved in Lactophenol solution	100 mL

Composition of Lactophenol

Phenol	20 mg
Glycerol	33.3 mL
Lactic acid	1.68 mL
Distilled water	20 mL

3.3.3. Methods for fungal isolation (Willoughby and Roberts, 1994; Lilley *et al.*, 1998)

For fungal isolation the lesions which have not yet completely ulcerated are most suitable. For isolation of fungus the following techniques were adopted.

1. The scales around the periphery of the lesion were removed and underlying skin was seared with a red hot spatula to sterilize the surface.
2. The anesthesized fish pinned on a dissecting tray was then taken to a laminar flow cabinet.
3. The underlying muscle of the lesion was then exposed by cutting the superficial tissues.
4. The affected underlying muscles were then excised into 4mm pieces and placed on a petridish containing the isolation medium.
5. Inoculated media were incubated at 25^{0}C and examined under phase contrast inverted microscope and first transfer was given after 6 h.

6. The emerging hyphal tips were repeatedly transferred to fresh plates of GP medium containing antibiotic until the cultures were free of bacterial contamination.
7. The isolates were then subcultured on GP agar and transfer was given at an intervals of 5 days.

3.3.4. Methods for sporulation of isolated fungus

For sporulation of fungus an agar plug containing actively growing mycelium was placed in a petridish containing GPY broth and was incubated for 4 days at about 20^{0}C. The resulting mat was washed by sequential transfer through 5 petridishes containing autoclaved pond water finally was and left overnight at 20^{0}C in autoclaved pond water. After about 12 hours motile secondary zoospores were observed under microscope.

3.3.5. Microscopical examination of isolated fungus

A portion of the ulcer tissue was taken and smeared on a clean glass slide. The smear was stained with cotton blue and was observed under the microscope.

Similarly, fungal hyphae and sporangium from the pure culture maintained in and GPYA, were observed under microscope after staining with cotton blue and measured with standard ocular micrometer.

3.3.6. Effect of temperature on the growth of isolated fungus

The fungal isolates were cultured at different temperatures from 20^{0}C –37^{0}C to observe its growth in GPA and GPYA media.

3.4. Bacterial cultures

3.4.1. Source of culture

Three types of bacteria, R1, R2 (fluorescent pseudomonad) and R3 (*Aeromonas caviae*) were isolated in our laboratory from infected air breathing fish of North Bengal (Pal and Pradhan, 1990, Pradhan 1992) and

were maintained routinely. These bacteria were used for different experimental works. Bacteria were also isolated from different infected fish.

3.4.2. Composition of bacterial media

Various types of culture media were used during this study. The names and composition of these media are given below:

Nutrient Broth

Peptone	100 gm
Beef extract	10 gm
Nacl	5 gm
Distilled Water	1000 mL

The ingredients were dissolved in distilled water. The pH was adjusted to 8.0-8.4 with 10(N) NaOH and steamed for 10 minutes. It was then filtered and pH was adjusted to 7.2-7.4. with sulphuric acid. The medium was sterilized at 115^{0}C for 20 minutes.

Nutrient Agar

Nutrient agar was prepared by adding 2% agar powder to the final nutrient broth solution. The agar was melted by heating the medium before sterilization.

Glucose Peptone Broth (GPB)

Glucose	3 gm
Peptone	1 gm
$MgSo_4.7H_2O$	0.128 gm
KH_2PO_4	0.014 gm
$CaCl_2$	8 mg
$FeSO_4$	0.5 mg
$MnSO_4$	0.5 mg
$CuSO_4.5H_2O$	0.1 mg
$ZnSO_4$	0.1 mg
Distilled water	1000 mL

All the ingredients were dissolved in distilled water and after dispensing into test tubes and 100 mL conical flasks (containing 30ml of broth) was sterilized at 115°C for 20min.

Nitrate Broth

KNO_3	1gm
Nutrient Broth	100 mL

Potassium Nitrate was dissolved in nutrient broth and distributed into test tubes containing inverted Durham's tubes filled with the medium and sterilized at 115°C for 20min.

Gelatin Agar

Gelatin	4g gm
Distilled water	50 mL
Nutrient agar	1000 mL

The gelatin was soaked in water for 30min and added to the molten nutrient agar. It was then mixed and sterilized at 115°C for 20 minutes and distributed into sterile petridishes.

Glucose-Phosphate medium

Peptone	5 gm
K_2HPO_4	5 gm
Distilled water	1000 mL
Glucose	5 gm

The first two materials were steamed with distilled water until the solids were dissolved, then filtered and the pH was adjusted to 7.4. Glucose was then added and 5ml volumes of medium was distributed into tubes. It was then sterilized at 115°C for 10 minutes.

Hugh and Leifson's of medium (Hugh and leifson, 1953)

Peptone	2 gm
NaCl	5 gm
K_2HPO_4	0.3 gm
Agar	3 gm

Distilled water 1000 mL

Bromothymol blue, 0.2% aquous soln. 15mL

The solids were dissolved by steaming. The pH was adjusted to 7.1, filtered and the indicator was added. Then it was sterilized at 115^{0}C for 20 minutes.

The carbohydrate solution was sterilized separately, cooled to 45^{0}C and mixed to the OF media to give a final concentration of 1%. It was then distributed asceptically into sterile tubes.

Medium A of King (King *et al.,* 1954)

Peptone	20 gm
Glycerol	10 gm
K_2SO_4 anhyd	10 gm
$MgCl_2$ anhyd	1.4 gm
Agar	20 gm
Distilled water	1000 mL

The constituents except agar were dissolved by steaming and the pH was adjusted to 7.2. The agar was then added and dissolved. The medium was sterilized by autoclaving at 121^{0}C for 10min.

Medium B of King for Fluorescin (King *et al.,* 1954)

Proteose peptone	20 gm
Glycerol	10 gm
K_2HPO_4	1.5 gm
$MgSO_4$, $7H_2O$	1.5 gm
Agar	20 gm

Distilled water 100 mL

The pH was adjusted to 7.2.

Procedure as stated for medium A was followed

Modified motility media (Hajna, 1950)

Peptone	10 gm
Beef Extract	3 gm
NaCl	5 gm
Agar	4 gm
Gelatin	80 gm
Cystein	0.2 gm
Ferrous ammonium sulphate	0.2 gm
Sodium citrate	2 gm
Distilled water	1000 mL

Gelatin was soaked in water for 30 minutes. The other ingredients were added and heated to dissolve and sterilized at 115^{0}C for 20 minutes.

Arginine Media

Peptone	1 gm
NaCl	5 gm
K_2HPO_4	0.3 gm
Phenol red, 10% aq. Soln.	1.0 mL
L(+) Arginine hydrochloride	10 gm
Agar	3 gm
Distilled water	1000 mL

The solids were dissolved in the distilled water and the pH was adjusted to 7.2. The medium was distributed into tubes to a depth of about 20mm and sterilized at 121^{0}C for 15 min.

Stains

Carbol fuchsin

Phenol -	85 gm
Basic fuchsin	15 gm
Ethanol	250 mL
Distilled water	1250 mL

The phenol and basic fuchsin powder were mixed and heated gently to dissolve the phenol. Ethanol and distilled water were added and filtered with Whatman filter paper. To carbolfuchsin weak 1 volume of strong carbol fuchsin were mixed (10-20) time volume of distilled water.

Gram stain

Crystal violate

Burke's Iodine

Saffranin

Crystal violate stain

Crystal violet	2 gm
95% alcohol	20 mL
1% aqueous ammonium oxalate solution	80 mL

Burke's Iodine

Iodine	1 gm
Potassium iodide	2 gm
Distilled water	1000 mL

Saffronin

Nitrite regent

Solution A

0.33% sulphanilic acid was dissolved gentle in 5 N acetic acid.

Solution B

0.5% a-naphthylamine was dissolved by gentle heating in 5N acetic acid.

10% Zinc dust suspended in 1% methylcellulose solution (Steel & Fisher, 1961).

Kovac's regent

ρ-dimethylaminobenzaldehyde	5 gm
Amyle alcohol	75 mL
Concentrated HCl	25 mL

The aldehyde was dissolved in alcohol by gently warming in water bath (about 50-55^{0}C). Then it was cooled. HCl was added with care. It was stored at 4^{0}C protecting from light.

Methyl red

Methyl red	0.04 gm
Ethanol	40 mL
Distilled water	100 mL

The methyle red was dissolved in the ethanol and diluted with the distilled water.

Acetylmethyl carbinol

5% α-naphthol solution 0.6 mL 40% potassium hydroxide (KOH) aquous solution 0.2 mL.

3.4.3. Methods for isolation of bacteria

The ulcerated area of the diseased fish was dissected out asceptically from the fish and then surface sterilization was done with 0.1% mercuric chloride. The dissected tissues were placed in 100ml conical flasks containing 15 ml nutrient broth medium supplemented with glucose. The flasks were incubated at 30^{0}C for 72 hours. The tissues were then removed and the cultures were observed under microscope. Then the cultures were diluted by serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) and bacteria were isolated by poor-plate method. Single separated colonies on agar plates were selected and streaked in nutrient agar slants and incubated for 24 hours at 30^{0}C. Thus isolated pure cultures were obtained. Code names were given to

each of the isolates and stored at 4^0C for characterization and identification of bacteria Barrow, G.I. and R.K.A. Feltham (1993) was followed.

3.5. Morphological and biochemical tests for bacterial identification

Shape of Cells

A drop of cell suspension of the test organism was placed on a clean grease-free slide, air dried and stained with carbol fuchsin and was observed under microscope. Diameter was measured with a standard ocular micrometer.

Motility

Tubes of motility medium were stab inoculated to a depth of about 5 mm to detect the motility. The tubes were incubated at 28^0C and the turbidity pattern of the medium was observed for 5 days. The bacterial cultures in nutrient broth were also observed under a phase contrast microscope.

Gram staining

Smears of test organisms were made from 24 hour old culture on nutrient agar slant on clean grease free glass slide.

The smears were air dried heat fixed and flooded with crystal violet stain for 1 minute. Slides were washed in 95% ethanol, which was poured drop-by-drop holding the slides in slanting position against white background till no colour came out from the lower edge of the slides. After washing with tap water these smears were stained with saffranin for 1 minute and rinsed in distilled water. The slide were air dreid and observed under microscope.

Production of catalase

Organisms from 24 hours old cultures were incubated for 24 hours on a slope of nutrients agar and 1 ml of 3% H_2O_2 solution was poured down the slope. Immediate evolution of gas indicate the presence of catalase activity.

Production of Oxidase

Organisms from 24 hour old cultures grown on nutrient agar (glucose free) were taken and smeared across a filter paper moistened with freshly prepared 1% tetramethyl-p-phenylenediamine dihydrochloride with a glass rod. The appearance of a dark purple colour within 30 second indicated a positive reaction

Oxidation or Fermentation of glucose

Oxidation or fermentation of glucose was done by inoculating dupliate tubes of Hugh and Leifson's medium containing glucose as the carbohydrate, by stabbing. After inoculation, sterile molten paraffin was poured on to the top of the tubes to a depth of 10 cm to seal it from air. The other tubes was left open. The tubes were incubated at 30^{0}C and observed for 14 days. If the blue colour of the medium changes to yellow in the open tube only, it indicated acid production from glucose by oxidation. If both tubes changes to yellow it indicated that glucose can also be broken down fermentatively (Huge and Leifson, 1953).

Acid and gas production in media containing different carbohydrates

The medium used to detect acid production from carbohydrates was a modified Hugh and Leifson's medium without agar. The following carbohydrates were tested: D-glucose, D-fructose, D-sucrose, D-Maltose, D-sorbitol, manitol, adonitol meso-inositol, L-arabinose. The liquid medium was inoculated and incubated at 30^{0}C for 14 days. If the colour of the medium changed from blue to yellow, it indicated acid production. To test for gas production inverted Durham's tubes filled with the medium was inserted into the broth. The gas, when produced, was accumulated at the top of the inverted Durham's tube

Nitrate reduction

Test tubes containing nitrate broth and filled in inverted Durham's tubes were inoculated and incubated for 5 days. The presence of nitrate (after reduction of nitrate to nitrate) was tested by addition of 1 mL of nitrite reagent

A (0.8% Sulphanalic acid in 5N Acetic acid) followed by 1 mL of reagent B (0.5% a napthylamine in 5N Acetic acid). Appearance of red colour indicated presence of nitrite. Zinc dust was added to the culture tubes showing negative test for nitrite appearance of red colour indicated presence of nitrate in culture tubes that was not reduced to nitrite by the test organisms. Any gas production in the Durham's tubes was noted.

Indole production

Tryptone broth was inoculated and incubated at 30^0C for 48 hours. Inddole prodction was tested by adding 0.5ml of Kovac's reagent and examined for 1min. Appearance of a red colour in the regent layer indicated indole production.

Methyl red reaction

Glucose phosphate medium was inoculated and incubated at 30^0C for 5 days. Two drops of methyl red solution was then added, shaken and examined. A red colour at the surface shown the positive M R reaction.

Acetylmethylcarbinol production: the Voges-Proskauer test

After completion of the methyl red test 0.6ml of 5% α-naphthol solution in ethanol and 0.2 mL of 40% potassium hydroxide aqueous solution was added and well shaken. The tubes were then held in a sloping position to increased the area of the air liquied interface and examined after 15 minutes and 1 hour. A strong red colour shows positive V.P. test .

Gelatin Hydrolysis

Gelatin agar plates were inoculated and incubated for 3 days. The plates were then flooded with 30% trichloroacetic acid, clear zones indicated areas of glatin hydrolysis.

Gelatin hydrolysis was also tested in the modified motility medium along with testing of motility and H_2S production. The medium was stabbed inoculated with a straight wire, inoculated at 28^0C and observed daily for 30 days for presence of liquefaction.

Argining hydrolysis

The arginine agar was inoculated by stabbing with a straight wire and a layer of sterile molten paraffin was poured with a pipette to a depth of about 2cm. The tubes were incubated at 30^{0}C and observed daily for 5 days for a colour change of the medium from yellow orange to red.

H_2S production

Tubes of the modified motility medium containing cystein and ferrous ammonium sulphate wre stabbed inoculated with the bacteria. A lead acetate paper was inserted between the cotton plug and the tube and inocubated at 28^{0}C. The tubes were examined daily for blackening of the media and the paper.

Pigment production

To test pigment production by Pseudomonads, King, Ward and Raney's media (A and B) were used. After inoculating, medium A was incubated at 30^{0}C for 24 to 96 hours and examined daily for pigment production. Medium B was incubated at 30^{0}C for 24 hours followed by room temperature (22-25^{0}C) for 72 h. To test for pigment production by coccus, organisms were grown on nutrient agar plates at room temperature and were kept under diffused day light.

3.6. Inoculation of fishes with bacterial suspensions

3.6.1. *Channa punctatus*

Intramuscular injection of 0.5 mL/100 g body weight (6-8 x 10^9 cells/mL) of bacterial culture, in pure (R1, R2 and R3) and in mixed culture of three bacteria (R1, R2 and R3) were given to *Channa punctatus.* Control fishes received only nutrient broth. For each set of experiment ten fishes were taken and each experiment was done five times. The gradual development of ulcer after injection was carefully observed. Mortality caused by the injection of bacterial culture was recorded up to 10 days of injection.

3.6.2. *Cyprinus carpio*

Intramuscular injection of 1mL/100 g body weight (6-8 x 10^9 cells/mL) of bacterial culture, in pure (R1, R2 and R3) and in mixed condition of three bacteria (R1, R2 and R3) were given to *C. carpio.* Control fishes received only nutrient broth. For each set of experiment 5 fishes were taken and each experiment were done four times. The gradual development of ulcer after injection was carefully observed. Mortality caused by the injection of bacterial culture was recorded up to 10 days of injection.

3.7. Inoculation of fishes with zoospore suspension

Healthy *C. punctatus* (20 – 30 g) were collected from the ponds of Sonapur. After bringing to the laboratory, the fishes were acclimatized in the laboratory condition in the glass aquarium at 25± 1^0C for 15 days and then 10 fishes were injected spores suspension of *Aphanomyces* sp. (F_{cs1}) at the rate of 0.5 mL/100 g of body weight intramuscularly as described by Roberts *et al.* (1993). The control set received only sterile saline (0.85% NaCl) solution @ 0.5 mL / 100 g of body weight. Simultaneously, five sets of experiment, each set consisting of ten fishes, were carried out.

3.8. Pathogenicity test of bacterial isolates

Attempts were made to determine the pathgogenicity of the isolated bacteria from *C. striata* and *Labio rohita*. For this purpose healthy fishes were collected from ponds of Sonapur, of the Darjeeling district that did not have any previous report of EUS outbreak. The fishes weighing about 20-30 g were brought to the laboratory and acclimatized in the glass aquarium measuring 90 x 35 x 35 cm. Water temperature was maintained at 28 - 30^0C. Intramuscular injection was given with 0.05 ml of cell suspension containing 3 – 5 x 10^7 cell cells / ml of each isolate (C_{s1}, C_{s2}, C_{s3}, C_{s4}, C_{s5}, L_{r1}, L_{r2}, L_{r3}, L_{r4} and L_{r5}). The control set received sterile saline (0.85% NaCl) solution @ 0.5 mL/100 g of body weight of the fishes. For each set of experiment ten fishes were taken and each experiment was repeated five times under identical conditions.

3.9. Inoculation of fishes with bacterial suspension (R1, R2, R3 and mixed culture of R1, R2 and R3 for histopathological studies

Healthy fishes (*Cyprinus carpio*) each weighing about 30 – 50 g were inoculated by intramascular injection with 0.85% saline suspension of R1, R2 and R3 bacteria in pure and mixed condition. The concentration of bacterial suspensions was $1x10^7$ cfu/mL. Five fishes were injected with each bacterial suspension and mixed suspension. The control set of ten fishes received 1.00 mL sterile saline solution. The fishes were observed daily for appearance of symptoms. The moribund fishes from each aquarium were taken out and sacrificed for histological studies. Two fishes from control set were also sacrificed and the tissue from liver, kidney and spleen were processed for histological studies. Histological sections were prepared and stained with Hematoxylene-Eosin stain.

3.10. Inoculation of fishes with mixed suspension of fungal zoospore and bacteria (R1, R2 and R3) in pure condition

Healthy fishes (*Channa punctatus*) each weighing about 20 – 30 g were inoculated by intramascular injection with 0.85% saline suspension of mixed R1 and zoospore, R2 and zoospore and R3 and zoospore. Ten fishes were injected with each bacterial suspension and mixed suspension. The control set of ten fishes received 0.05 mL sterile saline solution. Each experiment was repeated five times. The fishes were observed daily for appearance of symptoms. Nature of ulcer formation and mortality was recorded upto 15 days.

3.11. Histopathology of the experimentally infected fish with isolated fungus

Healthy *C. punctatus* (20 – 30 g of body weight) were inoculated with zoospores of isolated *Aphanomyces* sp. @ 0.5 mL/ 100 g of body weight. The fishes with ulcer were sacrificed after five days of inoculation for

histopathological observation. The sections of different organs of the experientially infected fishes were prepared and stained with Haematoxylin-eosin, Grocott stain (GMS) and PAS stain.

3.12. Materials for histopathological techniques

The ulcer tissue and other internal organs of diseased and healthy fish were fixed in Bouin's fixative and preserved in cedarwood oil if necessary. For histological studies sections of 6µ thickness were done after preparing the paraffin block and were stained with haematoxylin and eosin, Grocott hexamine (methenamine) silver stain and Periodic Acid Schiff's stain.

Bouin's fixative

Saturated aqueous picric acid solution	75 mL
Formalin	25 mL]
Glacial acetic acid	5 mL

Materials for Grocott hexamine (methenamine) silver staining for fungi (Gomori, 1946; Grocott, 1966)

Solution (a) 5 percent aqueous sodium tetraborate, i.e. Borax soln.

Solution (b) 5 percent silvernitrate in distilled water-5 mL

3 percent methenamine or hexamethylentetramin in distilled water 100 mL

Silver nitrate was added to the methenamine solution and shaked until the precipitation with first forms dissolved.

This mixture was kept for 1-2 months at 4^0C

Solution (c) Incubating solution

Borax solution	5 mL
Distilled water	25 mL
Methenamine silver soln. B	25 mL

The methenamine silver / water solution and the borax were heated to 56^{0}C and then mixed prior to use as the silver solution to degenerate when the borax was added.

Solution (d) Arzac's counter stain

Orange Green	0.25 gm
Light Green	1.00 gm
Phsphotungstic acid/	
Tungstophosperic acid	0.50 mg
50% alcohol	100 mL
Glacial acetic acid	1.25 mL
Sodium metabisulphate	1%
Gold chloride	0.1%
Sodium thiosulphate	3%
Chromic acid	5%

Materials for periodic acid schiff stain (PAS)

(i) **Basic fuchsin solutin**

(a) Basic fuchsin	0.1gm
(b) 95% alcohol	5.00 mL
(c) Water	95.00 mL

(ii) **Zinc (Sodium) hydrosulfite solution**

(a) Sodium hydrosulfite (Sodium dithionite)	1.0 gm
(b) Tartaric acid	0.5 gm
(c) Water	100 mL

(iii) **Light green stain**

(a) Light green	1.0 gm
(b) Glacial acetic acid	0.25 mL
(c) 80% alcohol	1000 mL

3.13. Methods of Histopathological techniques

3.13.1. Sampling and fixation

Live fish were quickly transferred to a container of benzocain solution (25 mg/L.). After two minutes, the fish was removed from the solution and pinned on a dissection tray. The external lesions were first excised from the fish body and immediately placed in Bouin's fixative. The internal organs were carefully dissected out from the body cut into small pieces of 3 to 5mm and immediately placed in the fixative. All tisues were kept in a volume of fixative at least 20 times the volume of the tissue and kept overnight.

3.13.2. Processing

The fixative was washed out of the sample by 70% alcohol. The tissue were subjected to routine processing (Schaperclaus 1986). Dehydration of the tissue was done by passing through a graded serried of alcohol solutions (70%, 90% and 100%). The tissue was then placed in xylene and infiltration was done with molten paraffin (melting point 58-60^{0}C) and allowed to cool harden. Sometimes tissues were preserved in cedarwood oil followng fixation and dehydration.

3.13.3. Sectioning

The hardened paraffin containing the tissue were trimmed into rectangular block, mounted on microtome and sections of 6μ thickness were cut. The sections were placed on grease free slides with albumin serving as an adhesive. The section were stretched on warm distilled water and the slides were dreid by keeping them overnight at room temperature.

3.13.4. Staining

Before staining the sections stretched slides were completely dewaxed by placing in xylene for 5 to 10 minutes.

3.13.5. Procedure for Haematoxylin – Eosin stain

After dewaxing the slides with stretched sections were transferred to absolute alcohol for 5 minutes and then through descending grades of alcohol e.g. 90%, 70%, 50% and 30% to water. The sections were then stained with haematoxylin and washed thoroughly with water to remove the excess stain and observed under the microscope to check the differentiation level. The nuclei were stained blue. The sections were then passed through ascending grades of alcohol and stained with alcoholic eosin. The excess eosin was washed in 90% alcohol and again observed under the microscope to check the differentiation level. If the counterstaining was satisfactory, the tissues were further dehydrated in absolute alcohol, cleared in xylene and mounted in DPX mountant.

3.13.6. Procedure for Grocott hexamine (methenamine) Silver staining for fungi

1. After dewaxing the sections were hydrated through graded alcohols to water.
2. Then the sections were oxidized in 5 percent aqueous chromium trioxide (chromic acid) for 1 hour.
3. The sections were then washed in tap water and rinsed in 1 percent sodium metabisulphite solution.
4. The sections were rinsed in distilled water and placed in preheated (56°C) incubating solution in the dark, upto 1 hour.
5. The sections were rinsed well in distilled water and toned in 0.1 parcent gold chloride for 4 minutes.
6. Then the sections were rinsed in distilled water and fixed in 3 percnet sodium thiosulphate, for 5 minutes.
7. Then the sections were countrstained inArzac's stain for 15-30 seconds.
8. The sections were then blotted, dehydrated and mounted in D.P.X. mountant.

3.13.7. Procedure for periodic acid-schiff stain (PAS)

1. The sections were rinsed in 100% alcohol after dewaxing.
2. Then the sections were brought down gradually to distilled water and immersed in 1% periodic acid for 10 munites.
3. The sections were then rinsed in tap water for 5 to 10 minute.
4. Then the section were immersed in Basic fuchsin solution for 2 minutes and then rinsed in tap water for 30 seconds.
5. The sections were then placed in sodium hydrosulphite solution for 30 minutes.
6. The sections were then rinsed in tap water for 3 to 5 minutes and then placed in light-green stain for 2 minutes.
7. Then the sections were rinsed for a short time in tap water
8. The section were then dehydrated in 96% alcohol for 10 seconds and in 100% alcohol for 1 minutes.
9. The sections were then rinsed twice in xylol for about 1 minute each time and mounted in D.P.X. mountant.