3. Materials and Methods

3.1: Plant materials

3.1.1: Collection of post harvest diseased fruits and vegetables from different markets of North Bengal

The northern part of West Bengal commonly called as North Bengal is endowed with diverse natural resources. The most important economic plantation crop of the region is tea. Other than tea the region is also famous for cultivation of orange, tomato and pineapple. It has been noticed that fungi and bacteria often attack fruits and vegetables. This fungal and bacterial attack happens during harvesting, transportation and marketing. The attack in the fruits by the microorganism may be primary or secondary in nature. In the present study 37 fungi and 76 bacteria have been isolated respectively from 9 different vegetables (potato, brinjal, tomato, cucumber, papaya, carrot, been, pees, onion) and 6 different fruits (pineapple, orange, banana, apple, mango, guava) samples. While performing Koch's postulates it has been observed that three different fruits and vegetables (orange, tomato and pineapple) were affected significantly. Although several fungi and bacteria attack the fruits and vegetables but three fungi and three bacteria out of 13 fungi and 29 bacterial isolates of 3 different fruits and vegetables (orange, tomato and pineapple; Table: 3.1) were selected for the present study. Selection was made on the basis of pathogencity of the bacteria and fungi performed during initial study.

3.1.2: Collection of plants for extraction of botanicals

Plants were selected on the basis of availability in the North Bengal (sub Himalayan West Bengal). Fresh disease free leaves of fifty different plant species belonging to different families were collected. The extracts of fifty species of plants belonging different families have been evaluated for their antifungal and antibacterial activity. A list of the plants has been presented in the table (4.1) (Rastogi & Mehrotra, 1995; Chatterjee & Pakrashi, 1997; Chopra *et al.*, 1996). Voucher specimens have been deposited in the Departmental Herbarium of the Department of Botany, University of North Bengal.

3.2: Fungal and bacterial cultures used

3.2.1: Source of fungal and bacterial culture:

Various fungal and bacterial pathogens were isolated from the infected orange, tomato and pineapple of North Bengal. Infected fruits and vegetables were collected from August 2005 to February 2008. Samples were collected from different markets where fruits are temporarily stored before distribution to different places for marketing. Ten samples of each fruits were collected. Collected samples were kept carefully and transferred to the plant pathology laboratory, Department of Botany, University of North Bengal. Orange was collected from the Garubathan market of Jalpaiguri. Tomato was collected from the Market of Haldibari, Cooch Behar. Pineapple was collected from the wholesale market of Bidhannagar, Darjeeling. Thirteen fungal and Twenty-nine bacterial isolates were found from these fruits.

3.2.1.1: Isolation of fungal culture

The infected samples were rinsed in distilled water and infected portions were cut into small pieces and surface-sterilized with 0.1% $HgCl_2$ for 1 min, rinsed in sterile distilled water and placed on agar plate in an incubator at 28±1^{0}C. The solidified plates incubated for 4-5 days at 28±1^{0}C temperature. Preliminary isolations were done in potato dextrose agar (PDA) supplemented with antibacterial streptomycin (Stevens, 1981). Isolated fungal colonies grown in the plates were picked up by inoculating needle and were placed in sterile PDA slants. The cultures were observed under microscope and pure cultures were sub cultured for further use. The pure cultures were maintained in refrigerator at 4-8^{0}C.

3.2.1.2: Isolation of bacterial culture:

The infected area of the diseased fruits were cut aseptically following Pal and Pradhan (1990) and placed in a conical flask containing 15 ml of nutrient broth. The flask was incubated at 32±1^{0}C for 72 hrs. Then the cultures were serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) and 1 ml of each dilution was added to 20 ml nutrient agar in 90 mm diameter sterile Petri plates and incubated at 32±1^{0}C for 24 hrs. Colonies grown on the agar plates were selected and then streaked on to nutrient agar slants to incubate at 32±1^{0}C for 24 hrs. All the isolates were given code names and stored at 4°C. For routine

experimental works, the isolates were sub-cultured by growing in nutrient broth for 24 hrs at 32±1^{0}C.

Table 3.1: Isolated bacterial and fungal culture of orange, tomato and pineapple

Fruits and vegetables	No of different bacterial organisms	No of different fungal organisms	Collection place
Orange	9	4	Garubathan , Jalpaiguri
Tomato	11	5	Haldibari, Cooch Behar
Pineapple	9	4	Bidhannagar,Darjeeling

3.2.2: Verification of the disease through Koch's postulates and Pathogenicity

3.2.2.1: Fungal pathogen

For verification of a particular pathogen Koch's postulates were done. Fresh fruits were collected from market and surface sterilized by dipping the fruits in 1% sodium hypochlorite for two minutes and washed in sterile distilled water. Scratches were made on the peel of the host fruits. Each scratch was 10 mm long and approximately 2 mm deep and on the circumference of the fruits and at equidistant places. Finally the fruits were inoculated with the conidial suspension following the inoculation technique of Dhingra and Sinclair, 1995. After 4 days of inoculation, when disease symptoms appeared on the fruits, the pathogen was re-isolated from infected fruits. For re-isolation, infected portions of the fruits were cut into small species (1-2cm), washed thoroughly with the sterile distilled water, surface sterilized with 0.1% Mercuric Chloride ($HgCl_2$) for 1-2 minutes, washed thrice with sterile distilled water and finally transferred aseptically into a sterile PDA slants. The inoculated slants were incubated at 28±1^{0}C and were observed till sporulation. Sporulated cultures were used for microscopic studies. The organisms were confirmed after comparing them with the respective stock cultures and coded accordingly. If an organism was consistently re-isolated then it was treated as a pathogen and was identified in the laboratory or elsewhere as mentioned in table (3.3).

Thirteen fungal isolates were found from 3 different infected fruits and vegetables. They were coded accordingly and have been shown in the table (3.2). Out of the 13 fungal isolates of orange, tomato and pineapple, *Fusarium moniliforme* (orange isolate), *Alternaria alternata* (tomato isolate) and *Alternaria alternata* (pineapple isolate)

were selected for the future studies, shown in the table (3.3) Selection was done on the basis of severity of damage done by the bacteria to the respective fruits.

Table 3.2: Isolated fungal cultures from infected pineapple, tomato and orange of North Bengal

Fruit and vegetables	Code name of fungal culture	Total no of fungal cultures
Orange	OF1-OF4	4
Tomato	TF1-TF5	5
Pineapple	PF1-PF4	4

Table3.3: Selected pathogenic fungal cultures isolated from infected pineapple, tomato and orange of North Bengal

Fungal culture*	Source	Identified by	Identification no
Alternaria alternata (AaP) [§]	Naturally infected pineapple	Dr. T. Prameda Devi; Dr. Nita Mathur	7063.08 (IARI, New Delhi)
Alternaria alternata (AaT) [§]	Naturally infected tomato	Dr. T. Prameda Devi; Dr. Nita Mathur	7065.08 (IARI, New Delhi)
Fusarium moniliforme	Naturally infected orange	Dr. T. Prameda Devi; Dr. Nita Mathur	7344.09 (IARI, New Delhi)
Fusarium moliniforme	Naturally infected orange	Dr. T. Prameda Devi; Dr. Nita Mathur	7434.09 (IARI, New Delhi)

[§]Codes of the pathogen have been presented in parentheses. *Fungal cultures used as test pathogen throughout the present study.

3.2.2.2: Bacterial pathogen

For verification of a particular pathogen Koch's postulates were done. Fresh fruits were collected from market and surface sterilized by dipping the fruits in 1% sodium hypochlorite for two minutes and washed in sterile distilled water. Scratches were made on the peel of the host fruits. Each scratch was 10 mm long and approximately 2 mm deep and on the circumference of the fruits and at equidistant places. Finally the fruits were inoculated by bacterial pathogen following the inoculation technique of Dhingra

and Sinclair, 1995. After 4 days of inoculation, when disease symptoms appeared on the fruits, the pathogens were re-isolated from infected fruits. For re-isolation, infected portions of the fruits were cut into small species (1-2cm), washed thoroughly with the sterile distilled water and finally transferred aseptically into a sterile NA slants. The inoculated slants were incubated at 32 ± 1^0C and were observed till colony appeared. Microscopic studies were performed of all the bacterial cultures. The organisms were confirmed after comparison with that of original stock cultures. If an organism was consistently re-isolated then it was treated as a pathogen and was identified in the laboratory or elsewhere.

Twenty-nine bacterial isolates were found from 3 different infected fruits and vegetables. They were coded accordingly and have been shown in the table (3.4). Out of the 29 bacterial isolates of orange, tomato and pineapple, three bacteria were selected for the future studies, shown in the (Table: 3.5). Selection was done on the basis of severity of damage done by the bacteria to the respective fruits.

Table 3.4: Bacteria isolated from infected orange, tomato and pineapple of North Bengal

Fruit and vegetables	Code name of bacterial culture
Orange	OB1-OB9
Tomato	TB1-TB11
Pineapple	PB1-PB9

Table 3.5: Selected pathogenic bacteria cultures isolated from orange, tomato and pineapple

Culture	Source	Identified by*	Bacterial culture
OB5	Naturally infected pineapple	Dr. A. Saha	*Xanthomonas* sp
TB3	Naturally infected tomato	Dr. A. Saha	*Pseudomonas syringae*
PB8	Naturally infected pineapple	Dr. A. Saha	*Erwinia* sp

OB5, TB3, PB8 were used as test pathogen throughout the present study.
*following Bergey's manual of determinative bacteriology and biochemical tests performed in the laboratory.

3.2.3: Source of antagonist fungal and bacterial culture:

3.2.3.1: Isolation of fungi from soil

Collection of samples: Soil samples were collected from the rhizosphere of different plants from nine tea gardens of the sub-Himalayan region of west Bengal during 2005 to 2008. For isolation of fungi the spread plate method was followed.

Preparation of dilution series: One gram of soil sample was weighed and dissolved in 10ml of sterile distilled water in a sterile test tube. From this 10 ml solution 1 ml was dispensed in another test tube containing 9 ml of sterile distilled water to make 10^{-1} dilution. Subsequently, the solution was further diluted to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}.

Spread plate technique: 100µl aliquot of soil dilution sample (10^{-1} to 10^{-7}) inoculated in PDA plates following spread plates technique and the plates were incubated at 28±1^{0}C until fungal colonies developed. Pure culture was obtained after placing 4mm fungal mycelia discs from plates on fresh PDA plates are incubated for 7-10days till the fungal mycelia covered the whole plate (Table: 3.6). Pure cultures were ascertained after microscopic observations of morphological characters of spores and mycelia.

Table 3.6: Fungi cultures isolated from soil

Location	Code name of bacterial culture	Total no of fungal cultures isolated
BAGDOGRA	SF1-SF7	07
MALBAZAR	SF08-SF13	06
KALCHINI	SF14-SF19	06

Isolation of antagonistic fungi: Soil isolates (Fungi) were screened for their antimicrobial activity if any by dual culture technique against three fungal pathogens [*Fusarium moniliforme; Alternaria alternata* (AaT) and *Alternaria alternata* (AaP)]. Details of the two selected fungi have been presented in the table (3.7). On the basis of preliminary studies 2 fungal antagonists (*Trichoderma harzianum* (SF1), *Aspergillus flavus* (SF7) were selected for their good antimicrobial properties.

Table 3.7: Selected fungal antagonists used during the study

Name of the fungal and bacterial antagonist	Source	Identified by	Identification no
Trichoderma harzianum	SOIL	Dr. T. Prameda Devi; Dr. Nita Mathur	ITCC-4572 IARI, New Delhi
Aspergillus flavus	SOIL	Dr. T. Prameda Devi; Dr. Nita Mathur	7435.09 IARI, New Delhi

3.2.3.2: Isolation of bacteria from soil

Source and Collection of soil: Rhizosphere serves as a source of large number of microorganisms. Therefore, in the present study, the rhizosphere was chosen as a source of soil for isolation of antagonistic microorganisms if any. Soil samples were collected from Bagdogra (Mango rhizosphere), Malbazar (Maize rhizosphere), Kalchini (Citrus rhizosphere) the three different agro climatic zones of sub-Himalayan West Bengal. In all the cases soil samples were collected in sterilized petriplates and stored at 4°C for further use. For isolation of bacteria the soil dilution and plate count method was followed.

Preparation of dilution series: One gram of soil sample was dissolved in 10 ml of sterile distilled water in a test tube. From this stock solution 1 ml solution was dispensed in another test tube containing 9 ml of sterile distilled water to make a dilution of 10^{-1}. In the similar way a dilution series from 10^{-1} to 10^{-5} were prepared.

Spread plate technique: 100µl of soil solution was poured into soil extract agar plate and spread by a glass spreader under aseptic condition. The plates were incubated at 32 ± 1^{0}C for 24 hours and the plates were observed for isolation of colonies.

Isolation of single colonies: From the plates 31 bacterial isolates (SB1 – SB31) were found (Table: 3.8). The isolated colonies were maintained on NA slants at 4°C and sub cultured at regular intervals.

Table 3.8: Bacterial cultures isolated from soil

Location	Code name of bacterial culture	Total no of bacterial cultures isolated
BAGDOGRA	SB01-SB08	08
MALBAZAR	SB09-SB17	09
KALCHINI	SB18-SB31	14

Isolation of antagonistic bacterial species: Bacterial isolates were screened for their antimicrobial properties by dual culture technique against the pathogens of orange, tomato and pineapple. On the basis of preliminary studies 2 bacterial antagonists (SB1 and SB2) were selected for good antimicrobial properties. The details of the two bacteria have been given in the table (3.9). These bacteria were also used for preparation of bio-formulations which were used for *in vivo* experiments.

Table 3.9: Selected bacterial antagonists used during the present study

Bacterial culture*	Source	Identification done following biochemical studies/ molecular studies
Lysinibacillus sphaericus (SB1)	soil	Both by biochemical and Molecular studies (following 16S rDNA sequence studies)
Pseudomonas sp. (SB2)	soil	By biochemical studies

3.2.4: Maintenance of stock cultures

Freshly prepared sterile PDA/NA slants were used for the maintenance of the fungal/bacterial cultures. The fungal/ bacterial cultures were also maintained in PDB/NB. Pathogens and antagonists were grown on sterile PDA/NA media were stored in two different conditions, *viz.* at low temperature in refrigerator (at 4°C) and at room

temperature. At the interval of two weeks subculture was done for preparation of inoculums for different experiments.

3.3: Characterization of the isolated bacteria

To identify the bacteria, a number of physiological and biochemical tests (Barrow and Feltham, 1993) were conducted following the identification scheme described by Carnanhan *et al.* (1991).

3.3.1: Morphological characterization

3.3.1.1: Shape and size of the bacteria: To examine the shape and size of the cells, a drop of cell suspension of the test bacterium was placed on a clean grease-free slide, air dried and stained with carbol fuchsin and observed under microscope. Diameter was measured with standard ocular micrometer

3.3.1.2: Morphology of the colony: Morphology of the bacterial colony on nutrient agar plates like texture (smooth or rough), appearance (glistering or dull) etc were examined.

3.3.1.3: Motility: To detect the motility of sample bacteria, tubes of modified motility medium were stab inoculated to a depth of about 5 mm. The tubes were incubated at 32 ± 1^0C and the turbidly pattern was observed for 5 days.

3.3.2: Physiological and Biochemical tests

3.3.2.1: Gram Staining: The smears of isolates were made on separate glass slides. Smears were allowed to air dry and heat fixed. The smears were covered with crystal violet for 30 seconds. Each slide was washed with distilled water for a few seconds, using wash bottle. Each smear was covered with iodine solution for 30 seconds. The iodine solution was washed off with 95% ethyl alcohol. Ethyl alcohol was added drop by drop, until no more colour flows from the smear. The slides were washed with distilled water and drained. Safranin was applied to the smears for 30 seconds, washed with distilled water and blotted dry with absorbent paper. The stained slides were observed under microscope to study the Gram character and cell morphology of the bacterial isolates.

3.3.2.2: Indole Test: Test tubes were inoculated with bacterial isolates and one tube was kept as an uninoculated comparative control. Tubes were incubated at 37 ± 1^0C for 48 hours. After 48 hours incubation 1 ml of Kovac's reagent was added to each tube including control. The tubes were gently shakened after intervals of 10-15 min. The test tubes were allowed to stand to permit the reagent to come to the top.

3.3.2.3: Methyl Red and Voges –Proskauer Test: MR-VP tubes were inoculated with isolated cultures and 1 tube un-inoculated was kept as control. All tubes were incubated at 32±1^{0}C for 48 hours. After incubation media was divided equally into two tubes for each isolate. To one of the tube marked as MR (for Methyl Red) 5 drops of Methyl red indicator was added. To the other tube marked as VP, 12 drops of VP reagent I and 2-3 drops of V-P reagent II were added. Tubes were shaken gently for 30 seconds. The reaction was allowed to complete for 15-30 minutes.

3.3.2.4: Citrate Test: Simmon's Citrate agar was inoculated with isolated cultures by streaking and 1 tube was kept as uninoculated control. All the slants were incubated at 32±1^{0}C for 48 hours. Observation of color change from green to blue indicated positive result.

3.3.2.5: Casein Hydrolysis: Milk agar plates were inoculated with bacterial isolates as a single streak and one plate was kept as control. Plates were incubated for 24-48 hrs at 32±1^{0}C in an inverted position. Formation of a clear halo around the bacterial line of inoculation indicated positive result.

3.3.2.6: Catalase Test: The cultures were inoculated on NA slants and incubated at 32±1^{0}C for 24-48 hrs. After that, 3-4 drops of 10% hydrogen peroxide was allowed to flow over the growth of each slant culture.

3.3.2.7: DNase activity: Test cultures were inoculated on the surface DNase plate by streaking and incubated at 32±1^{0}C for 36 h. The plates were observed for the formation of a pinkish/clear halo around the bacterial growth.

3.3.2.8: Gelatin liquefaction test: Each gelatin containing tubes were inoculated with loopful of isolates. They were incubated at 32±1^{0}C for 2 days. The cultures were then allowed to chill by keeping them in ice for 15 minutes. The tubes that contained liquid media even after chilling were positive for gelatin liquefaction.

3.3.2.9: Nitrate reduction: Nitrate Broth was inoculated and incubated for 48 hrs. 1 ml of nitrite reagent A followed by 1 ml of reagent B was added. A deep red colour showed the presence of nitrite and thus showed that nitrate had been reduced and indicated a positive reaction. To tubes, not showing a red colour within 5 min powdered Zinc was added and allowed to stand. Red colour formation confirmed the presence of nitrate in the medium (i.e. not reduced by the organism).

3.3.2.10: ONPG Test: Tubes of ONPG broth were inoculated and incubated for 48 hours. β-galactosidase activity was indicated by the appearance of a yellow colour due to the production of o-nitrophenol.

3.3.2.11: Oxidase activity: A fresh solution of the reagent was prepared each time of use by adding a loop full of tetramethyl-*p*-phenylenediamine dihydrochloride to about 3 ml of sterile distilled water. A filter paper disc was soaked in a sterile plastic Petri dish with a few drops of the indicator solution and the test culture was smeared across the moist paper with a platinum loop. The appearance of a dark purple colour on the paper within 30 seconds denoted a positive reaction.

3.3.2.12: Phenylalanine decarboxylase: Tubes containing Phenylalanine agar media were inoculated with bacterial isolates and incubated at 32±1^{0}C for 24-48 hrs. The tubes were then acidified with 0.1 N HCl drop by drop until the medium was yellow. Then 0.2 ml of a 10% aqueous solution of $FeCl_3$ was added, shaken and observed for any colour change. A positive reaction was indicated by a dark green colour which quickly fades.

3.3.2.13: Starch hydrolysis: Starch agar plates were inoculated with the bacterial pathogen and antagonists by single streak. The plates were incubated at 32±1^{0}C for 48 hours in an inverted position. The plates were then flooded with iodine solution with a dropper for 30 seconds. The excess iodine solution was poured off.

3.3.2.14: Acid production in TSI agar

TSI slants were inoculated with test organism and incubated for 24hr at 30C and observation was recorded.

3.3.2.15: Tween 80 hydrolysis (Lipid hydrolysis): The test cultures were streak-inoculated on the surface of Tween 80 Nutrient Agar plates and incubated at the optimal growth temperature of the organism and observed for each day. An opaque halo of precipitation around the growth indicated hydrolysis of Tween 80.

3.3.2.16: Urease Test: Plates containing urea agar medium were inoculated by streaking and incubated for 24-48 hours at32±1^{0}C. Colour change of the media from yellow to pink around the bacterial inoculation line indicated positive result.

3.3.2.17: Sugar fermentation:

All bacterial strains were tested for sugar fermentation to check for their ability to utilize sugar as carbon source and to produce acid. The peptone water broth was inoculated with bacterial culture and incubated for 24-48 hours. The indicator Phenol Red remains red at neutral pH but turns colourless at acidic pH. Thus, the colour change of the broth culture from red to yellow confirms positive result for sugar fermentation and if remains red, is a negative result. The test was repeated with the same medium but using different sugars in each case. The sugars used were glucose,D-arbinose, xylose, fructose, galactose, maltose, sucrose, raffinose, lactose, dulcitol and rhamnose.

3.4: Pathogenicty

Initially fruits of different varieties or of different places were inoculated by three selected pathogenic fungi and three selected pathogenic bacteria separately. Healthy fruits without injuries and infections were selected. Fruits were dipped in the solution of 1% sodium hypochlorite solution for 2 minutes and then rinsed with sterile distilled water and air dried in a sterile atmosphere (in a laminar air flow). Wounds were made in fruits (orange, tomato and pineapple) by sterile scalpel instead of pin as used in pinprick method (Singh and Mandal, 2006).

Tomatoes and oranges: Ten tomatoes (each 5 cm in diameter) and ten oranges (each 7 cm in diameter) were wounded by a sterile scalpel at equidistant spots on the equatorial regions of the fruits. Altogether ten scratches were made on the peel of the fruits. Each scratch was 10 mm long and approximately 2 mm deep. Tomatoes were inoculated with *Alternaria alternata* (isolate AaT at conidial concentration of $5x10^4$ spores /ml) and bacterial suspension (of *Pseudomonas syringae*.) separately. Oranges were inoculated with *Fusarium moniliforme* (at conidial concentration of $1x10^5$ spores /ml) and with bacterial suspension (*Xanthomonas* sp.). The tomatoes and oranges were kept at 20^0 C for 6 days. During incubation the trays were covered by transparent polythene sheets, perforated in some places for aeration. Separate control sets were maintained where sterile distilled water was sprayed instead of conidial suspension/bacterial suspension.

Pineapple: Five pineapples (each 12 cm in diameter and 18 cm in length) were taken for each set and two different sets were kept in two different sterile glass chambers.

One set was kept as control where only sterile distilled water was sprayed. Each pineapple was scratched at twenty different equidistant spots on the circumference of the fruits in two rings. Size of the spots and inoculation procedures etc. were similar with that of oranges and tomatoes. Pineapples were inoculated with *Alternaria alternata* (isolate AaP at conidial concentration of $1x10^4$ spores /ml) and bacterial suspension (*Erwinia* sp.) separately. The glass chambers containing pineapples were kept at 25°C for 6 days. During incubation the glass chambers were covered by transparent polythene sheets, perforated in some places for aeration.

Assessment of disease: Disease was assessed by area of damaged tissue by transparent graph paper method. A graph paper was photo copied on a transparent sheet and the sheet was used to measure the diseased area. Diseased area was drawn a separate transparent sheet and was placed on the transparent graph sheet. The number of small squares of the graph paper covered by the diseased area was multiplied by the defined area of each small square and thus the area was determined. Area of damaged tissue (brown to black necrotic area around the scratched portion) were recorded individually and sum total of damaged area in 100 scratched spots after inoculation and desired incubation were recorded.

3.5: Extraction of botanicals

3.5.1: Aqueous extract:

Following the method of Mahadevan and Sridhar (1982) with some modifications, the extracts of the plant parts were made. Fresh plant materials were collected and washed thoroughly with sterile distilled water and allowed to dry at room temperature. After drying the materials were weighed, ground and extracted separately with sterile distilled water (0.5g/ml). The extracts were filtered through double-layered cheese cloth and then centrifuged at 20,000 rpm for 15 minutes for multiple times until clarification as suggested by Kagale *et al.* 2004. The supernatants of the aqueous extracts were sterilized by passing through a Millipore filter (0.2μm). All extracts were stored at 4^0C. The extracts were used for spore germination bioassay and TLC plate bioassay.

3.5.2: Alcohol extract:

Fresh plant parts were washed thoroughly with sterile distilled water and surface water were soaked by blotting paper at room temperature. The material (5gm) was

ground in mortar or pastel with 6 ml 50% ethanol with autoclaved water to make 0.5g/ml concentration. The extracts were filtered through double-layered muslin cloth and centrifuged at 10,000 rpm for 30 min. The supernatant of alcohol extracts were collected in plastic vial and all extracts were stored at 4^0C until used for bioassay.

3.5.3: Dry extract

Plant parts were dried, ground to a fine texture, and then soaked in desired solvent for extended periods. The slurry was then filtered after which it was dried under reduced pressure (In Rotary vacuum evaporator) and re-dissolved in the alcohol or in the desired solvent to a determined concentration. Crude products were then used in spore germination bioassay, disc diffusion bioassay, agar cup bioassay, poisoned food technique and TLC plate bioassay for antifungal properties and in a variety of experiments to screen for antifungal properties of plants.

3.5.4: Isolation of phytochemicals from selected plants for their antifungal and antibacterial activity

The crude extract shall be fractionated by different methods including silica gel column chromatography using various different solvent system following standard methods (Harborne, 1973; Saha *et al.*, 2005b; Evans, 2002). The fraction will be monitored using Thin Layer Chromatography. The exact procedure of each extract will depend on the chemical constituents of that extract.

3.5.4.1: Chemical extraction by soxhlet

A fine powder of dried leaves was extracted in different solvent through soxhlet for 24hrs in each extract. Soxhlet is equipment which is used to exhaustively extract chemicals in different solvent. Different solvent was used according to increasing polarity of solvent. 3 different extract was prepared by using suxhlet apparatus model HP-6-500 and concentrated by rotary evaporator model Heidolph. Benzene was used 1st and then extract was re -extracted by chloroform and methanol respectively. Dry leaves powder of *Xanthium strumarium* and *Datura stramonium* (150g) was taken and placed in soxhlet column and run for 20hrs using benzene solvent. The extracted fraction was checked by spore germination bioassay and then followed by chloroform and methanol respectively.

3.5.4.2: By chemical fraction method:

Ten gram fresh leaf was air dried and dried powder was mixed thoroughly in 100ml solvent mixture of methanol: water (4:1) ratio. After 5 min it was filtered by filter paper in a flask. Thus, two fraction aqueous phase and residue were separated. The aqueous phase was evaporated up to 1/10 the volume at 40^0C the evaporated solution acidified by adding H_2SO_4 and three volume chloroform, separated by separating funnel into two flasks, aqueous acid layer and chloroform layer. The chloroform extracted layer had moderately polar extract such as terpenoids/phenolic compounds. The aqueous acid basified to 10ph with NH_4OH and then dissolved in chloroform-methanol (3:1) ratio in twice volume and again separated. The aqueous basic may have the alkaloids. The residue formed during first filtration was mixed with ethyl acetate (5volume) and then filtered, one filtered residue may have polysaccharide and other may have fat wax or neutral compounds. Each chemical fraction was checked by spore germination bioassay, 30 ml of spore suspension was placed in glass slide and added 30 ml chemical fraction. From the preliminary results it was found that terpenoids/phenolic compounds fractions [through routine separation of different bioactive fractions following Wagner and Bladt, (1996)] of selected plants were effective in controlling pathogens. Hence, effective bioactive fraction (terpenoids/phenolic compounds) along with crude fraction was considered during the determination of MIC as stated in details in the experimental section of the present study.

3.5.5: Spore germination bioassay

Ten days old sporulated fungal culture was taken and approximately 3-5ml sterile distilled water was poured in the culture tube aseptically. Gentle scrapping was done by an inoculating needle on the agar surface. After the scrapping, the tube was shaken and the resultant mixture was strained through cheesecloth. The filtrate was used as spore suspension. The concentration of the spores in the suspension was adjusted by adding sterile distilled water following hemocytometer count.

Along with the aqueous leaf extract, inhibitory effects of different fractions (5 in no) obtained after routine separation were subjected to bio assay against three *F. moniliforme* and *A. alternata* isolates (AaT and AaP both) were tested. The spores of the pathogens were allowed to germinate in sterile distilled water drops mounted on

sterile grease free slides kept in a humid chamber in case of control. In experimental sets plant extract (30 µl) was placed on the centre of a grease free microscopic slide and allowed to evaporate. After evaporation of the solvent (ethanol), spore suspension was mounted on the slides in the same place where the extract was applied. In solvent control set fresh solvent (50% ethanol) was placed and subsequently evaporated before application of spore suspension. The slides were then incubated at 28 ± 1^0C in a humid chamber. Two small glass rods (60 mm in length) were placed in a 90 mm petridish and a slide was placed on the rods in a uniformly balanced position. Sterile distilled water was carefully poured in the petridish so that the bottom of the slide remained just above the water surface. The petridish was then covered and incubated at 28 ± 1^0C. Following 48 h of incubation, the slides were stained with lacto phenol-cotton blue and observed under the microscope. Approximately, 200 spores were observed in each slide for germination. The entire experiment was repeated thrice.

3.5.6: Bioassay by poisoned food technique

Two milliliter of plant leaf aqueous extract was added to 18 ml of the molten PDA medium mixed well and poured in sterile petridish (90 mm diameter) under aseptic condition and was allowed to solidify. In control sets 2 ml of sterile distilled water was added instead of plant extracts. The both experimental and control plates were inoculated with the pathogen and incubated for required period. Radial growth of the pathogen was measured.

3.5.7: Agar cup method for detection of anti-microbial activity by *Xanthium strumarium* and *Datura stramonium* and determination of MIC

For screening of inhibitory effect of botanicals against test pathogens, the spore germination bioassay technique and agar cup bioassay technique was followed. Minimum inhibitory concentration (MIC) of all active components was measured following standard protocols (Suleman *et al.*, 2002; Saha *et al.*, 2005a).

A sensitive and quick microplate method as suggested by Eloff (1998) was followed to determine the minimal inhibitory concentration of plants extracts for bacteria and fungi. Minimum inhibitory concentration (MIC) was determined by the micro dilution method using serially diluted plant extracts. Various concentrations (example. 0.10µg/ml, 0.20µg/ml, 0.40µg/ml, 0.50µg/ml and 0.100µg/ml etc) of the plant extracts

were prepared. Nutrient agar was used for the growth of bacterial strain and potato dextrose agar was used for the growth of fungi. By means of a cork borer (5 mm in diameter) a cup or well was made in a seeded (either by bacterial suspension or by fungal spore suspension) agar plate. The cup or well was filled with 50µl plant extract and was incubated in the incubators meant for bacteria or fungus as applicable. The extract diffused from the cup to a certain extent and inhibited the growth of the pathogen if it contained any antimicrobial properties. It was found that the diameter of the inhibition zone was more when the concentration of the antimicrobial properties was more. In the 1st phase crude plant extract was used for the detection of antimicrobial activity. In the 2nd phase bioactive fraction was used for the detection of antimicrobial activity. The plates containing bacteria were incubated at 37±1°C for 24 hr and the plates containing fungi were incubated at 28±1°C for 48hrs. The antimicrobial activity was noted on the basis diameter of growth inhibition zone.

3.5.8: Bioassay of fungicides:

Bioassay of fungicides was done by taking five different concentrations of two different fungicides following poisoned food technique as described by Suleman *et al.*,(2002). Minimum inhibitory concentration (MIC) was determined by the micro dilution method using serially diluted plant extracts. Various concentrations of the fungicides were prepared. Potato dextrose agar was used for the growth of fungi. By means of a cork borer (5 mm in diameter) a cup or well was made in a seeded (either by bacterial suspension or by fungal spore suspension) agar plate. The cup or well was filled with 50µl fungicides and was incubated in the incubators. The fungicides diffused from the cup to a certain extent and inhibited the growth of the pathogen if it contained any antimicrobial properties. It was found that the diameter of the inhibition zone was more when the concentration of the antimicrobial properties was more. The plates containing fungi were incubated at 28±1°C for 48hrs. The antimicrobial activity was noted on the basis diameter of growth inhibition zone. The plates were incubated at 28 ± 2^0C. After the required incubation period, the radial growth of the mycelia was measured and percent inhibition over control was calculated. Minimum inhibitory concentration (MIC) value of the test fungicides was also determined.

3.5.9: Thin layer chromatography plate bioassay

TLC originally developed from a need to separate chemical mixture. In TLC, the stationary phase is coated as a thin (1.0 mm) layer on a glass or metal plate foil plate that is then placed in a reservoir of mobile phase. The various substances move from the spot at different rate and is deposited in the absorbent layer in the different position. The movement of a given analytic is characterized by retardation factor (R_f).

3.5.9.1: Preparation and activation of TLC plates

The stationary phase (silica gel G as slurry) was applied to glass plates, using a plate spreader (applicator) that made a uniform layer of 1 mm thickness. Glass plates (20cm×10 cm) were dipped into chromic acid to remove all dirt and grease from the glass plates. The glass plates were washed in distilled water and thereafter, they were arranged on a platform of 100cm x 20 cm length. Seventy gram silica gel G was added in 100ml distilled water, shaken vigorously and then poured in the slot of TLC applicator. A coat of silica gel (1mm thick) was applied on TLC plates using the applicator. Then the plates were dried at room temperature and kept for the further use.

TLC plates were activated at 70^0C for 45 minutes in a hot air oven. Activated plates were allowed to cool down at room temperature. The extracted samples were applied as spots at one end at 1.5cm from the edge of the plate. The sample loaded plates were kept in a glass chamber containing solvent [chloroform–methanol (9:1) and hexane-ethyl acetate- methanol (60:40:1) respectively]. When solvent moved up to 15cm from the point of sample application, plates were taken out from solvent chamber and air dried until solvent evaporated completely. Spore suspension was prepared using Richard's solution in place of distilled water and poured in an atomizer. The TLC plates were placed in upright position and sprayed with suspended spores in media. The plates were incubated in humid chamber for 48 to 72 hrs. The spores germinated on plates keeping clear zones of growth inhibition. The diameter and R_f values of inhibition zones were recorded if any.

3.5.9.2: Phytochemical analysis

Different extracts of plant samples were spotted (10µl containing 100µg/ml crude extract) on pre-coated silica gel 60 F_{254} aluminum TLC plates (Source: SRL, India, Silica gel 60 F_{254}; Particle size: – 325 Mesh). TLC plates were subjected to linear ascending of

solvent system of hexane: ethyl acetate: methanol in the ratios of 60:40:1 (v/v). When solvent moved up to 8 cm from the point of sample application, plates were taken out from solvent chamber and air dried until solvent evaporated completely. The chromatograms were dried by flowing hot air to remove the remaining solvents. The plates were then sprayed with different chromogenic spray reagents. The detection of phytochemical constituent was done under UV light ($\square_{max}$ at 254 nm and 365 nm) as well as by spray reagents. The spray reagents were prepared in laboratory by standard protocol (Wagner and Bladt, 1996).

3.5.9.2.1: Column Chromatography

Concentration of the extracts from the Soxhlet apparatus afforded a brown gummy mass, which was subjected to column chromatography over silica gel (Source: SRL, India; Particle size: 60-120 Mesh). Elution with appropriate solvents by a solvent gradient system with gradually increasing the polarity of solvent mixture afforded different fractions, which were concentrated by evaporation. Each fraction was analyzed by TLC plate bioassay. The semi-purified fraction showed antifungal activity in bioassay. The fraction was further purified by extensive column chromatography over silica and the major fraction (approx. 15 mg) was analyzed by UV-Vis, IR and NMR (^{1}H- and ^{13}C-) spectroscopy.

3.5.9.2.2: Spectroscopic analyses

UV spectroscopic analyses of the sample were done in UV-160A/UV-visible recording spectrophotometer, Shimadzu, Japan. IR analysis was done in Shimadzu-8300, Japan. Finally NMR (^{1}H- and ^{13}C-) spectroscopy was done in Bruker-AV 300, Germany.

3.5.10: *In vivo* application of botanicals in fruits and vegetables.

In vivo application of potential leaf extracts were done following the method of Hadizadeh *et al.* (2009) with some modifications. Healthy fruits were collected and surface sterilized as stated in case of pathogenicity (Section 3.4).

Tomatoes and oranges: Thirty tomatoes (each 5 cm in diameter) and thirty oranges (each 7 cm in diameter) were wounded by a sterile scalpel at equidistant spots on the equatorial regions of the fruits. Altogether ten scratches were made on the peel of the fruits. Each scratch was 10 mm long and approximately 2 mm deep. The leaf extracts were sprayed to fruits until runoff. Ten fruits in three sets were taken in three trays. One

set of fruits were kept 'scratched - uninoculated', second set was 'scratched – inoculated' and third set was 'scratched -treated (with leaf extract) and inoculated'. Tomatoes were inoculated with *Alternaria alternata* (isolate AaT at conidial concentration of $5x10^4$ spores /ml) and oranges were inoculated with *Fusarium moniliforme* (at conidial concentration of $1x10^5$ spores /ml). The tomatoes and oranges were kept at 20^0 C for 12 days and disease was assessed after 6 days intervals.

Pineapple: Five pine-apples (each 12 cm in diameter and 18 cm in length) were taken for each set and three different sets were kept in three different sterile glass chambers. Each pineapple was scratched at twenty different equidistant spots on the circumference of the fruits in two rings. Thus altogether 100 scratched spots were there for each treatment. Size of the spots and inoculation procedures etc. were similar with that of oranges and pineapples. Pineapples were inoculated with *Alternaria alternata* (isolate AaP at conidial concentration of $1x10^4$ spores /ml). The glass chambers containing pineapples were kept at 25°C for 12 days and disease was assessed at 6 days intervals.

Similar experiments were also performed with spray of pathogenic bacterial suspensions instead of fungal spores in all the three fruits.

Percent inhibition of damage was calculated in case of leaf extract 'treated and inoculated' fruits in comparison to untreated inoculated fruits by the formula as stated below:

[(Area damaged in untreated fruits - area damaged in pretreated fruits)/area damaged in untreated fruits]x 100.

3.6: Evaluation of antagonists

3.6.1: Dual culture test for screening and evaluation of antifungal fungal antagonist

Microbial antagonists were screened following the dual culture method of Johnson and Curl (1972) for their bio control potential. In this technique, both the test pathogen and bio control agents were grown simultaneously in the same plate. Mycelial discs (4 mm) cut from 15 days old cultures of the pathogen and of a biocontrol agent were placed aseptically into the peripheral region of a sterile PDA Petri plate (90 mm) in a straight line but opposite to each other and incubated at 28 ± 1^0C. Radial growth of the pathogen and the biocontrol agent were measured after the desired incubation period and

percent inhibition (in comparison to control where no biocontrol agent was placed) was determined for each experiment.

3.6.2: Dual culture test for screening and evaluation of antifungal bacterial antagonist

For *in vitro* screening of antagonistic bacteria against post harvest fungal pathogens dual culture technique was followed where a 4 mm diameter mycelial disc of fungal pathogen was inoculated at the centre of a petriplate (90mm diameter) containing PDA media and the 24-h-old culture of the test strain of bacterial isolate was streaked in a semicircle pattern at a distance of 4cm from the centre. A control plate was kept where only mycelia disc of fungal pathogen was inoculated. The plates were incubated at 28±2°C and checked for inhibition zones after every alternative day till the fungal growth in the control plate reached the edge of the plate.

3.6.3: Dual culture test for screening and evaluation of antibacterial bacterial antagonist

For *in vitro* screening of antagonistic bacteria against post harvest bacterial pathogens, dual culture technique was followed where a bacterial pathogen and antagonist bacterial strain were streaked perpendicularly at the centre of a petriplate (90mm diameter) containing NA media in such way that they did not touch each other. Inoculated plates were again incubated at 37±1°C for 48 h (Dubey and Maheshwari, 2002). A control plate was kept where only bacterial pathogen was inoculated.

3.6.4: Evaluation of crude culture filtrates of the antagonists

Culture filtrate of fungal antagonists was collected from 15 days old cultures grown in PDB in 100 ml Erlenmeyer flasks. Culture fluid was filtered through cheese cloth and finally centrifuged at 3000rpm and the supernatant was subjected to filter sterilization. One ml of filter sterilized culture supernatant was mixed with 9 ml of sterile molten PDA in a petridish and allowed to solidify. One mycelial disc of 4 mm in diameter was cut from the advancing zone of a culture (maintained in petridishes) was placed on solidified PDA medium at the centre. In control set 1 ml of sterile distilled water and 9 ml of molten PDA media was poured. The control plates were also inoculated with mycelial discs as mentioned in case of culture fluid supplemented petridishes. All the petridishes were incubated at 28±1 °C.

3.6.5: Molecular characterization of antagonist bacteria

3.6.5.1: Isolation of genomic DNA by CTAB method

Procedure: Cells were grown in Nutrient broth and incubated over night at 30° C at 120 rpm. The tubes were then chilled on ice for 5 min. Each 1ml culture was taken in 1.5ml microcentrifuge tubes, pelleted at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was re-suspended in TE buffer. Cell suspension was transferred to a clean centrifuge tube and Proteinase K (10mg/ml) and 10% SDS were added to it and mixed well. The mix was incubated at55°C for 16 hours. 1% CTAB in 1M NaCl and 5 M NaCl were added to the samples, mixed well and incubated at 65°C for 10 min. Next, the mixture was centrifuged at 12,000 rpm for 10 min at 4°C temperature. Then the clear solution was transferred to a clean eppendorf tube and cell debris was discarded. Then aqueous phase was transferred and 0.6 vol. 70% ethanol (chilled) was added to it. Then the microcentrifuge tubes were spinned at 12,000 rpm for 15 min. washing of pellet with 70% ethanol was repeated with addition of 2 volumes of ethanol and spinned at 10,000 rpm for 10 min. Then the supernatant was discarded and the pellet was allowed to dry for 5 – 10 min at room temperature and re-suspended in 50 µl of TE buffer.

3.6.5.2: PCR amplification of 16s rDNA using universal primers:

Total genomic DNA was isolated by CTAB method (Gomes *et al.* 2000). PCR amplification of the 16S rDNA gene of the antagonistic bacterial isolates was performed in 25µl reaction using the following conditions: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 49°C for 2 min, extension at 72°C for 2 min and a final extension at 72°C for 7 min on a thermal cycler (Applied Biosystems GeneAmp PCR 2400).

The primers used were: F1 (positions 8 to 27 of *E. coli* numbering system) 5'-**AGAGTTTGATCATGGCTCAG**-3'and R1 (positions 1509 to 1491 of *E. coli* numbering system) 5'- **GGTTACCTTGTTACGACTT**.-3' (Weisberg *et al.*, 1991). PCR product was resolved on 1% agarose gel containing ethidium bromide (0.5µg/ml) at 50V for 1 h in 1X TAE (Tris-Acetic acid EDTA) buffer. The amplified product was purified using PCR purification Kit (Bangalore Genei, Bangalore, India) and was sequenced at Bangalore Genei Sequencing Services. Similarity searches of the sequences

were carried out using the BLAST function of GenBank (Altschul *et al.* 1990; Tamura, *et al.* 2007).

3.6.6: Characterization of antimicrobial metabolites produced by the bacterial and fungal antagonists:

3.6.6.1: Cellulase (Kumar *et al.*, 2005): M9 medium agar amended with 10g of cellulose and 1.2g of yeast extract per litre of distilled water was used to test the cellulase activity. Wells were cut on 90mm diameter cellulose agar plates and to it 100µl bacterial culture filtrates were poured in the wells. In case of fungal antagonists 7 days old culture filtrate were poured instead of bacterial culture filtrate. The plates were incubated at 37°C for 8 days and observed for the formation of a clear halo after staining with Congo red solution (0.1%) followed by distaining with 1M NaCl solution at an interval of 1hr for 4 times. Plates were checked for the formation of clear halo around the wells that indicated positive result for Cellulase production.

3.6.6.2: Pectinase: 10g pectin and 1.2g yeast extract were amended in M9 medium agar and it was used for inoculation of bacterial strains or fungal blocks. The plates were flooded with 2M HCl after 2 days of incubation at 28°C. Clear halos around the colonies were considered as positive for Pectinase production.

3.6.6.3: Lipase: Lipase medium containing 10g peptone, 5g NaCl, 0.1g $CaCl_2$, 1ml Tween 80 and 20g agar dissolved in 1000ml of distilled water was used to check the lipase production. It was used for inoculation of bacterial strains and incubated over night. Clear zones around the colonies indicated positive result.

3.6.6.4: Phosphatase: Bacterial culture was streaked onto Pikovskaya's agar medium. Phosphatase activity was determined by the development of a clear zone in after 2-5 days of incubation of assay plates at 28°C. In case of fungal antagonists, mycelial blocks were inoculated onto Pikovskaya's agar medium. Phosphatase activity was determined by the development of a clear zone in after 2-5 days of incubation of assay plates at 28±1°C.

3.6.6.5: Chitinase: Chitinase activity was observed by the method of Kohli *et al.* (2002). Bacterial strain was grown overnight in nutrient broth and culture was centrifuged at 10,000 rpm for 15 min. In case of fungi fungal mycelia was grown for 96 hours in potato dextrose broth and culture was centrifuged at 10,000 rpm for 15min. Culture supernatants were filtered through 0.2µm diameter cellulose acetate filter paper (Sartorius) and 100µl of the filtered sample was used for detection of chitinase activity in M9 media supplemented with glycol chitosan . Positive result was indicated by the presence non-fluorescent lytic regions under UV light source. M9 media supplemented with glycol chitosan.

3.6.6.6: Detection of Microbial Siderophore (fungal):

The Chrome Azurol S (CAS) agar is a mixture of 4 solutions that were prepared and sterilized separately before mixing. Solution 1(Fe-CAS indicator solution) consisted of 10ml of 1mM $FeCl_3.6H_2O$ (in 10mM HCl), 50 ml of aqueous solution of CAS (1.21mg/ml) and 40ml of aqueous solution of HDTMA/CTAB (1.82mg/ml). Solution 2 (buffer solution) was prepared by dissolving 30.24gm of PIPES in 750ml of salt solution. The distilled water was added to bring the volume to 800ml after adjusting the pH to 6.8 with 50% KOH. The solution was autoclaved after adding 15 g agar. Solution 3 contained 2 g glucose, 2 g Mannitol, and trace elements in 70 ml distilled water. Solution 4 was 30 ml filter sterilized 10% (w: v) casamino acid. At 50^0C after autoclaving solution 3 and 4 were added to the buffer solution. Solution 1 was added last with sufficient stirring to mix the ingredients. This mixture yielded blue to dark green colour (Husen, 2003).

The greenish coloured medium was inoculated with a fungal block and incubated for 5-7 days. Orange halo around the fungal culture corresponds to positive result.

3.6.6.7: Detection of Microbial Siderophore (bacterial):

The CAS agar was prepared as mentioned in section -3.6.6.6. Bacteria cultures were inoculated on the surface of CAS agar plates and incubated overnight at 30^0C. Siderophore production was detected by the presence of orange halo around the colonies.

3.7: Scanning electron microscopy (SEM)

Preparation of sample for electron microscopy and observation:

Biocontrol bacteria were streaked in U-shaped manner at a distance of 40 mm diameter from the centre in a petriplate containing PDA. One circular mycelial block of 4 mm in diameter of the pathogen were carefully placed at the centre of the petriplate. One separate plate was also inoculated by the fungal pathogen and was treated as control. Both the plates were incubated at 28±1°C until the mycelia in the control plate (pathogen only) reached the edge of the petriplate. One cover slip was carefully placed between the fungus and bacteria (bacteria-fungus interaction zone of dual culture) in the experimental plate. The cover slip was removed after fungal mycelia grew over the cover slip and finally subjected to following treatment. Fungal mycelia were fixed with 2.5% glutaraldehyde solution for 1 hour. Glutaraldehyde was removed by slight decanting. Then 50% ethanol was added to the interaction zone on the cover glass and allowed for 5 minutes incubation. After 5 minutes 50% ethanol was replaced by 70% ethanol and it was also incubated for 5 minutes. In the similar way 70% ethanol was replaced by 90% ethanol. After 15 minutes again 90% ethanol was added and incubated for another 15 minutes. Finally the cover glass was dipped in absolute alcohol. After the absolute alcohol treatment the cover glass was air dried and coated with gold. Gold coating was performed IB2-ion coater (Japan). The cover glass was then observed in scanning electron microscope [Model : Hitachi S-530 (Japan) 1986] with appropriate magnification.

3.8: Statistical analysis

Some of the statistical analysis was done using Smith's statistical package (version 2.5). The package was developed by Dr. Gray Smith, Pomona College, Claremont-91711,USA. In some other cases statistical Package for the Social Sciences (SPSS), version 11.0, SPSS Inc., Chicago, Illinois were also used.

3.9: Preparation of Media chemicals and reagents

3.9.1. Chrome Azurol S (CAS) agar

3.9.1. 1. Solution 1(Fe-CAS indicator solution):

1mM $FeCl_3.6H_2O$ (in 10mM HCl)	10 ml
CAS solution (1.21mg/ml)	50 ml
HDTMA solution (1.82mg/ml)	40 ml

3.9.1. 2. Solution 2:

PIPES buffer	30.24 gm
Distilled water	750 ml
pH	6.8

3.9.1. 3. Solution 3:

glucose	2 gm
Mannitol	2 gm
Distilled water	70 ml

3.9.1. 4. Solution 4:

10% (w: v) casamino acid 30 ml (filter sterilized)

3.9.2: DNase Test media (Himedia):

DNase Test Agar	42.1g
Distilled Water	1000 ml

The ingredients was dissolved; pH was adjusted to 7.3, sterilized at 121°C for 15 min and distributed aseptically to sterile petriplates.

3.9.3. Gelatin Media:

Gelatin	12g
Peptone	0.5g
Beef Extract	0.3g
Yeast Extract	0.1g
Distilled water	100ml

The above mixture was heated to dissolve; and sterilized at 121°C for 20 min.

3.9.4. MR-VP broth:

Peptone	7.0 g
Potassium phosphate	5.0 g
Dextrose	5.0 g
Distilled water	1000 ml

The ingredients were steamed until the solids were dissolved, and adjust to pH6.9 and sterilized at 115°C for 10 min. Half of the media was used for VP tests.

3.9.5. M9 agar basic media:

Sodium hydrogen phosphate	6.0g
Potassium hydrogen phosphate	4.5g
Ammonium chloride	1.0g
NaCl	0.50g
$CaCl_2$	15.0mg
$MgSO_4.7H_2O$	245.0mg
Thiamine HCl	10.0mg
Yeast extract	1.2g
Distilled water	1000 ml

All the ingredients were heated to dissolve completely.

3.9.6. Nutrient Agar (HiMedia)

28gms of the media was suspended in 1000 ml of distilled water according to the manufacturer's protocol. It was boiled to dissolve the medium completely and sterilized by autoclaving at 15 lbs pressure (121°C for 15 min).

Peptic digests of animal tissue	5.0 g
Sodium chloride	5.0 g
Beef extract	1.5 g
Yeast extract	1.5 g
Agar	15.0 g
Final pH (at 25°C)	7.4±0.2

3.9.7. Nutrient Broth (Himedia)

13 gms was suspended in 1000ml of distilled water. It was boiled to dissolve the medium completely and sterilized by autoclaving at 15lbs pressure (121°C for 15 min).

Peptic digest of animal tissue	5.0 g
Sodium chloride	5.0 g
Beef extract	1.5 g
Yeast extract	1.5 g
Final pH (at 25°C)	7.4±0.2

3.9.8. Nitrate Broth:

KNO_3	1.0 g
Nutrient Broth	1000 ml

KNO_3 was dissolved in the nutrient broth and distributed into tubes containing inverted Durham tubes and sterilized at121°c for 15 min.

3.9.9. ONPG Broth:

ONPG	6.0 g
0.01M-Na_2HPO_4	1000 ml

ONPG (O-nitro-phenyl-D-galactopyranoside) was dissolved in the phosphate solution (pH-7.5) at room temperature and sterilized by filtration.

ONPG solution	250 ml
Peptone water	750 ml

ONPG solution was aseptically added to the Peptone water and distributed in 2.5 ml volumes in sterile test tubes.

3.9.10. Pikovskaya's Agar

Yeast extract	0.5 g
Dextrose	10.0 g
Calcium phosphate	5.0 g
Ammonium sulphate	0.5 g
KCl	0.2 g
$MgCl_2$	0.1 g
$MnSO_4$	0.0001 g
$FeSO_4$	0.0001g

Agar	15 g
Distilled water	1000 ml

All the ingredients were heated to dissolve completely and then sterilized at 121°C for 15min. the media was distributed into sterile petriplates.

3.9.11 Potato dextrose broth (PDB)

Peeled potato	40.0 g
Dextrose	2.0 g
Distilled water	100 ml

Peeled potato in required amount was boiled in distilled water. The potato broth was collected by straining through muslin cloth and then required amount of dextrose was added. Finally, the medium was sterilized at 15 lb p.s.i. for 15 minutes.

3.9.12. Potato dextrose agar (PDA):

Peeled potato	400.0 g
Dextrose	20.0 g
Agar	20.0 g
Tap water	1000 ml
pH	6.0

Peeled potato was cut into small pieces and boiled in required volume of tap water. The mixture was filtered through muslin cloth and the extract was mixed with dextrose and agar and heated in order to dissolve all the ingredients. The media was then sterilized at 121°C for 15min.

3.9.13: Peptone water:

Peptone	10.0 g
NaCl	5.0 g
Distilled water	1000 ml
pH	7.2-7.4

The solids were dissolved by heating in water and pH of the solution was adjusted to 8.0-8.4 and boiled for 10min, the mixture was filtered and adjusted to pH 7.2-7.4 and sterilized at 121°C for 15 min.

3.9.14. Phenylalanine decarboxylase Agar:

DL-Phenylalanine	2.0 g
Yeast extract	3.0 g
Na_2HPO_4	1.0 g
NaCl	5.0 g
Agar	20.0 g
Distilled water	1000ml

The ingredients were dissolved by heating in water, filtered and dispensed into tubes and then sterilized at 121°C for 15 min. Test tubes were solidified in a slanting position to give a long slope.

3.9.15. *Pseudomonas* Agar (For Fluorescein) (HIMEDIA):

37.3 g of the dehydrated medium along with 10.0 ml of glycerol was dissolved in 1000 ml of distilled water according to the manufacturer's protocol; the media was then heated and sterilized at 121°C for 15min and dispensed into sterile petriplates.

3.9.16. Richard's solution/medium (RM)

KNO_3	10.0 g
KH_2PO_4	5.0 g
$MgSO_4, 7H_2O$	2.5 g
$FeCl_3$	0.02 g
Sucrose	50.0 g
Distilled water	1000 ml

All the constituents were mixed with required amount of distilled water. Constituents were then dissolved by stirring and sterilized at 121°C and 15 lb p.s.i. for 15 minutes.

3.9.17. Soil extract agar (Cowan and Steel, 1993)

Preparation of soil extract: 5.0 g of soil was dried and sieved through fine mesh (No. 9). Air dried soil was suspended in 180 ml of distilled water and sterilized by autoclaving at 15 lbs pressure (121°C for 15 min) and allowed to settle down overnight (turbidity was removed by adding $CaCO_3$).Water was then removed by filtration through the Whatmann filter paper (Grade-II).

Peptone	5.0 g
Meat extract	3.0 g
Agar	20.0 g
Soil extract	1000 ml

The above ingredients were heated to dissolve completely, pH was adjusted to 5.4 (soil pH) and sterilized by autoclaving at 15 lbs pressure (121°C for 15 min). The media was dispensed into sterile petriplates.

3.9.18. Simmon's Citrate agar:

$(NH_4)HPO_4$	1.0 g
K_2HPO_4	1.0 g
NaCl	5.0 g
Sodium citrate	2.0 g
$MgSO_4$, $7H_2O$	0.2 g
Bromothymol blue	0.08 g
Agar	15.0 g
Distilled water	1000 ml
pH	6.9

The solids were dissolved by heating and then distributed into test tubes and then tubes were sterilized at 121°C for 15 min.

3.9.19. Skim milk agar:

Skim milk powder	100.0 g
Agar	15.0 g
Distilled water	1000 ml

Skimmed milk and water were taken in a corked bottle & agar and water were taken in a separate bottle. Then both were autoclaved at 121°C for 15 min. The two mixtures were added to each other, mixed well and poured into sterile petriplates.

3.9.20. Starch agar:

Starch (soluble)	20.0 g
Peptone	5.0 g
Beef extract	3.0 g
Agar	15.0 g

Distilled water	1000 ml
pH	7.0

The above mixture was heated to dissolve; and sterilized at 121°C for 20 min.

3.9.21.Triple Sugar Iron (TSI) Agar:

Meat extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g
Glucose	1.0 g
Lactose	10.0 g
Sucrose	10.0 g
$FeSO_4.7H_2O$	0.2 g
NaCl	5.0 g
$Na_2S_2O_3.5H_2O$	0.3 g
Agar	20.0 g
Distilled water	1000ml
Phenol red (0.2% aq. soln)	12 ml

The mixture was heated to dissolve the solids in water and the indicator solution was added, mixed and dispensed into tubes. The media was sterilized at 121°C for 15 min and cooled to form slopes with deep buts, about 3cm long.

3.9.22. Tryptone broth:

Tryptone	10.0 g
Sodium chloride	5.0 g
Calcium chloride (1 M)	1.0 g
Distilled water	1000 ml

3.9.23. Tween 80 media:

Peptone	10.0 g
NaCl	5.0 g
$CaCl_2.2H_2O$	0.1g
Agar	20.0g
Distilled water	1000 ml

The ingredients were dissolved by steaming and pH was adjusted to 7.4. It was sterilized at 121°C for 15 min and cooled to 40-50°C.

Tween 80 was filter sterilized and 10 ml of it was added aseptically to flask to give a final concentration of 1% and then dispensed into petriplates.

3.9.24. Urea media

Peptone	1.0 g
NaCl	5.0 g
KH_2PO_4	2.0 g
Agar	20.0 g
Distilled water	1000 ml

The solids were dissolved by heating and adjusted to pH 6.8, filtered and sterilized at 121°C for 15min.

Glucose	1.0 g
Phenol red, 0.2% aq. soln	6 ml

The above ingredients were added to the molten base, steamed for 1h and cooled to 50°C.

Urea, 20% aq. Soln. 100ml

Urea was sterilized by filtration and added aseptically to the base cooled at 50°C. The medium was aseptically distributed into sterile petriplates.

INDICATORS AND REAGENTS:

3.9.25. Methyl Red indicator:

Methyl red	0.1 g
Ethyl alcohol (95%)	300.0 ml
Distilled water	200.0 ml

Methyl red was dissolved in alcohol, distilled water was added and the mixture was filtered.

3.9.26. Phenol Red indicator:

Phenol red	0.2 g
Ethyl alcohol (95%	500.0 ml
Distilled water	500.0 ml

Phenol red was dissolved in alcohol, distilled water was added and the mixture was filtered.

3.9.27. Nitrate test reagents:

3.9.27.1 Solution A

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

3.9.27.2 Solution B

0.6% dimethyl-α-naphthylamine was dissolved in 5 N-acetic acid.

3.9.28. Oxidase test reagents:

1% α-naphthol in 95% ethanol.

3.9.29. Kovac's reagent:

p- dimethylaminobanzaldehyde	5.0g
Amyl alcohol	75 ml
Conc. HCl	25 ml

The *p*-dimethylaminobenzaldehyde was dissolved in alcohol by gentle warming in a water bath (about 50-55°C), cooled and then the acid was added to conc. HCl with care. The solution was stored at 4°C in dark.

3.9.30. Gram Staining Reagents:

3.9.30.1 Crystal violet	Micro Master Laboratories PVT LTD
3.9.30.2 Lugol's Iodine	Micro Master Laboratories PVT LTD
3.9.30.3 Safranine	Merck-India.

Preparation of spray reagents

3.9.31. Vanillin –sulphuric acid (VS)

3.9.31.1 ethanol vanillin (solution-I)	1%
3.9.31.2 ethanolic suphuric acid (solution-II)	10%

3.9.32. Catalase test reagent:

H_2O_2 Solution	3-6%

3.9.33. Voges-Proskauer test reagents:

3.9.33.1 α-naphthol solution (in ethanol)	5%
3.9.33.2 KOH aqueous solution	40%

3.9.34. Salkowski's Color Development Reagent:

$FeCl_3$	0.05 M
Perchloric Acid	35%

$FeCl_3$ was added to Perchloric Acid to produce the Salkowski's reagent.