PART- I

A) Studies on post-harvest fungal diseases

Infected fruits of different mango varieties like Alphanso, Malgoa, Pairi, Lalbagh, Totapuri, Langra, Neelum, Benishan, Kesar, Dasheri, Mallika and many local varieties were collected from different district of Marathwada region of Maharashtra state. Small pieces, measuring 2 mm each of infected tissue, were cut off from mango fruits with the help of sterile sharp knife. Pieces of diseased fruit were washed with tap water and surface sterilized with 1% Sodium hypochloride solution for 2 min, washed twice with sterilized distilled water and then dried using sterile filter paper. The pieces were separately transferred to sterilized petri-dishes containing potato dextrose agar (PDA) medium and incubated at 25°C for 10 days. Petri-dishes were observed daily and colonies of fungi were chosen. The isolated fungi were purified using single spore technique and then kept in a refrigerator on PDA medium (Gams et al., 1998). Pure colonies of fungal isolates were identified according to Ellis (1971). Symptoms were confirmed by Koch’s postulates.

B) Impact of physical factors on development of post-harvest diseases

Healthy mango fruits of Alphanso variety were collected from Aurangabad fruit market. Mango fruits were surface sterilized with 0.1 % HgCl₂, pricked to a depth of 2mm and washed with sterile distilled water. The injured fruits were dipped in spore suspension (10⁶ spore/ml) of test fungi for 2min. Then the fruits were placed in sterilized polythene bags as one fruit per bag. These polythene bags containing mango fruits were incubated at different temperature and R.H. percentage were maintained by the method recommended by Buxton and Mellanby (1934). Severity of rot
was recorded on 8\textsuperscript{th} day of incubation on the basis of percent fruit area infected.

Effect of temperature and R.H. on spore germination of test fungi was examined by placing spores on glass-slide placed to different levels of temperature and R.H. Effect of temperature and R.H. on action of cellulase and pectinase enzyme of the test fungi was investigated by incubating inoculated fruits at different temperature and R.H. at 25\degree C. On 8\textsuperscript{th} day of inoculation 5g of rotted tissue was macerated with distilled water and 0.5N NaCl. The extract was filtered and filtrate was centrifuged at 4000 rpm for 25min. the supernatant was used as enzyme sample. Pectinase was assayed gives in 2ml of enzyme sample, 5ml of 1% pectin dissolved in buffer solution (pH- 4.5), 1.8ml of phosphate citrate buffer solution (pH-4.0) and 1.5ml of distilled water. The cellulase was assayed using 2ml of enzyme sample, 5ml of 1% CMC (Carboxy Methyl Cellulose), dissolved in buffer solution (pH-4.5), 1.8ml of sodium citrate buffer (pH-4.8) and 1.8ml of distilled water. The enzyme action was assayed by determining loss in viscosity of the reaction mixture after 120 min at 30\degree C following the method of Bell et al. (1955).

C) Detection of fungal diseases by X-Ray scanning

To study the detection of fungal infection in mango fruits by X-ray scanning technique, the spore suspension of \textit{A. niger}, \textit{A. flavus}, \textit{A. fumigatus}, \textit{Colletotrichum gloeosporioides}, \textit{Penicillium digitatum} and \textit{Rhizopus stolonifer} were inoculated by sterilized disposable syringe in Kesar variety of mango fruits in aseptic condition. Healthy mango without inoculation was treated as control. After four days these mango fruits were scanned by digital X-ray system. The Specifications with which X-ray exposure was taken are as kv = 63; mA = 160 and mAs = 13.

D) Detection of spongy tissue of Alphanso mango by X-Ray scanning

Mango fruits cv. Alphonso was collected from local market. Spongy tissue was studied by cutting and X-ray scanning. The fruits were scanned for spongy tissue presence under X-ray imaging machine at. The
Specifications with which X-ray exposure was taken are as kv = 63; mA = 160 and mAs = 13.

E) **Growth pattern and culture characteristics of *Aspergillus niger* isolates**

Isolates of *Aspergillus niger* were isolated from mango fruits infected with *A. niger* rot from different places of Marathwada region of Maharashtra state. The single spore of isolates of the fungus was grown in GN broth media for seven days at 25°C. In order to study growth pattern and culture characteristics of *Aspergillus* isolates they were grown on solid and liquid GNA media. These isolates were incubated at room temperature, solid media for seven days and liquid media for two weeks and the results were noted in the form of growth pattern, colony color, mycelia weight and sporulation.

F) **Molecular characterization of *Aspergillus niger* isolates by ISSR DNA isolation**

Ten *Aspergillus niger* isolates were inoculated separately in glucose nitrate medium contained in 250 ml Erlenmeyer flasks and incubated at 25°C for 7 days in an orbital shaker (120 rpm). Mycelium was harvested by filtration through Whatman filter paper no.3 and immediately frozen in liquid nitrogen. The frozen mycelium was pulverized, freeze dried and ground to a fine powder using a sterile pestle and mortar. The mycelium powder was stored at -20°C until needed.

Isolation of genomic DNA from mycelial tissue of supplied *Aspergillus niger* was carried out by using modified method of Liu et al. (2000). 500 µl of lysis buffer was taken in 1.5 ml of eppendorf tube; to this 0.100gms of mycelia piece were added. These eppendorf tubes were mixed well and incubated at room temperature for 10 min. The tubes were vortex and incubated again at room temperature for 5 min. To this 150 µl of potassium acetate and glacial acetic acid was added. Again the tubes were vortexed briefly and spined at 10000 rpm for 5 min. The supernatant was collected in fresh MCF tube and the tubes were centrifuge again at 10000 rpm for 10
Materials and Methods

min. The supernatant was taken in new MCF tube and equal volume of chilled isopropanol was added to it. This mixture was again centrifuged at 10000 rpm for 1 min. The supernatant was discarded and the DNA was pelleted out by wash with 70% ethanol. The pellet DNA was dried and dissolves in TAE buffer and then store at 4°C. A small aliquot of isolated DNA was ran on a 1% (w/v) TAE gel to check the quality of DNA sample.

**ISSR Primers**

Following four primeres were used for amplification

- Primer 811 – 5’ GAG AGA GAG AGA GAG AC 3’
- Primer 812 – 5’ GAG AGA GAG AGA GAG GAA 3’
- Primer 814 – 5’ CTC TCT CTC TCT CTC TA 3’
- Primer 816 – 5’ CAC ACA CAC ACA CAC AT 3’

**PCR amplification of DNA with ISSR primers**

Amplification of DNA was performed in Finnzyme make Piko thermal cycler programmed for thirty eight cycles. Initial denaturation was carried out at 94°C for three minutes, annealing at 56°C and extension for one min at 72°C and five minutes for final extensions. PCR products were run and analyzed by electrophoresis using 1% agarose gel in 1X TBE buffer at 60V. Gel was externally stained with ethidium bromide at working concentration 0.5µg ml⁻¹. Gel was visualized under UV light, gel pictures were documented by using Biotron make gel documentation system.

**Band scoring and data analysis**

For each fragment that was amplified using ISSR primers was treated as a unit rearrangement in genome. The primers which were given scorable and consistently reproducible amplicons were considered. The gel pictures were taken and documented to computer by using alpha imager gel documentation system and size of each amplicons was measured by using alpha imager software with respect to standard molecular weight DNA ladder and molecular weight of each of the potential specific bands was calculated using the software program alpha imager.
**Phylogenetic analysis**

The dendrogram was plotted by using bioinformatics phylogeny free tree and tree view of DNA fingerprint analysis tool.

The phylogenetic analysis carried out by using free tree bioinformatics phylogeny tool of DNA fingerprint analysis based on amplicons sizes given by primers showed that isolate number 5 is the basic origin sample. Boot strapping analysis was carried out to find the original an-sister progeny analysis amongst these samples by using free tree bioinformatics tool of phylogeny analysis.
PART- II

A) Study of cellulase and pectinase enzymes

I) Cellulase

Production of cellulase

Production of cellulase was made by growing the fungi on liquid medium containing CMC – 10gm, KNO₃ – 0.25%, KH₂PO₄ – 0.1% and MgSO₄.7H₂O – 0.05%, pH – 5.0. Out of which 25ml of medium was poured in 100 ml Erlenmeyer conical flasks and autoclaved at 151bs pressure for 15 minutes. The flasks on cooling were inoculated separately with 1ml spore suspension of test fungi prepared from the 7 days old cultures grown on PDA slants. The flasks were inoculated for 6 days at 25 ± 1°C with diurnal periodicity of light. On the 7th day the flasks were harvested by filter the contents through Whatman filter paper no.1. The filtrates were collected in the pre-sterilized bottles and termed as crude enzyme.

Assay for cellulase

Cellulase activity was assayed by viscometric method as viscosity loss % after 60 minutes.

II) Pectinase

Production of pectinase

Production of pectinase was made by growing the fungi on liquid medium containing pectin – 10gm, KNO₃ – 0.25%, KH₂PO₄ – 0.1%, MgSO₄.7H₂O – 0.05%, pH – 5.0. Out of which 25 ml of medium was poured in 100 ml Erlenmeyer conical flasks and autoclaved at 15 lbs pressure for 20 minutes. The flasks on cooling were inoculated separately with 01 ml standard spores / mycelial suspension of test fungi prepared from 7 days old cultures grown on PDA slants. The flasks were incubated for 6 days at 25± 1°C with diurnal periodicity of light. On 7th day, the flasks were harvested by filtering the contents through Whatman filter paper no.1. The filtrates were collected in the presterilized bottles and termed as crude enzyme.
Materials and Methods

Assay for pectinase

Pectinase activity was assayed by viscometric method as viscosity loss % after 60 minutes.

B) Biochemical changes

Biochemical changes in mango pulp due to post-harvest fungi have been divided in to two parts. In first part biochemical changes due to five isolates of *Aspergillus niger* were separately studied because *Aspergillus niger* is very severe disease as compared to other diseases while in second part of biochemical changes, due to other post-harvest fungi Viz *Aspergillus flavus*, *A. fumigatus*, *Colletotrichum gloeosporioides*, *Penicillium sp.* and *Rhizopus stolonifer* were studied. In the first part of biochemical changes, spore suspension of five isolates of *Aspergillus niger* that is, *A. niger* isolate on Parbhani local variety (Asn1), *A. niger* isolate on Langra variety from Nanded (Asn2), *A. niger* isolate on Dasheri variety from Aurangabad(Asn3), *A. niger* isolate on Shundari variety from Parbhani(Asn4) and *A. niger* isolate on Benishan variety from Beed market (Asn5) were separately inoculated by sterilized disposable syringe in same aged mango fruits of five varieties that is Alphanso, Benishan, Dasheri, Kesar and Local in aseptic condition. Fruits of every variety without inoculation were kept as control. Methodology for second part of biochemical changes was same but in this type fungi were different. After seven days of incubation pulp was collected in separate sterilized containers and biochemical changes were estimated.

a) Estimation of dry matter (DM)

Dry matter (DM) was calculated by weighing the sample after drying to a constant weight in an oven at 95 ± 5°C. For this purpose, 100 gm of sample was taken in a clean dry pre-weighed tray and is kept in oven for 48 hours or more, till constant weight. Weight of the dried sample was reported as percent dry matter (DM).
b) **Estimation of cellulose**

Changes in cellulose content were carried out by method recommended by Sadashivam and Manickam (1992). One gram of sample was taken and to it 3 ml of acetic acid: nitric acid reagent was added. It was then mixed by using vortex mixer. After that it was placed in water bath at 100°C for 30 min and centrifuged for 15-20 min. Supernatant was discarded, residue was washed with water. 10 ml 67% H2SO4 was added to the residue left for one hour. After one hour 1 ml of that solution was diluted up to 100ml. To 1 ml of the diluted solution 10 ml of anthrone reagent was mixed well. Tubes were heated in boiling water bath for 10 min. It was then cooled and O.D. at 630 nm was measured. Blank was prepared with anthrone reagent and water. Standard curve was prepared by taking 0.4 to 2ml of standard cellulose solution (Corresponding to 40-200µg of cellulose), volume was equalized and anthrone reagent was added to develop the color as above. Then amount of cellulose was calculated from the standard graph.

c) **Estimation of pectin**

The pectin content was estimated following method recommended by Sadashivam and Manickam (1992). 5 gm of fruit pulp was taken into beaker to it 30 ml of 0.01N HCl was added and boiled up to 30 min and after cooling it was filtered with Whatman filter paper No.54 and collected the filtrate into conical flask and labeled them as 1. The residue was taken into beaker 10 ml of 0.05N HCL was added and boiled up to 20 min. and again filtered with the Whatman filter paper No.54, the filtrate was collected into another conical flask and numbered them as 2. The residue was again taken into beaker and 10 ml of 0.03 N HCL was added and boiled for 10 min. Then it was filtered with Whatman filter paper No.54 and washed with hot water and labeled the filtrate as no.3. Residues were kept aside.

The filtrate was collected into conical flasks and the volume was made up to 50 ml. 10 ml of aliquot was Pipetted out, 25 ml of water was
added and the acid was neutralized with 1N NaOH using Phenolphthalein as indicator. Then an excess of 1 ml of 1N NaOH was added with continuous stirring and it was incubated for overnight. 5 ml of 1N acetic acid was added and after 5 min, again 25 ml of 1N CaCl₂ solution was added with stirring. It was kept for 1 hrs and boiled for 2-3 min. After cooling, it was filtered by a pre-weighed Whatman filter paper no.1. The precipitate was washed with almost boiling water until the filtrate was free from chloride. Filtrate was tested with AgNO₃ for chloride. The filter paper with calcium pectate was dried overnight at 100 °C in weighing dish and weighed.

Pectin content as % calcium pectate was calculated by using following formula.

\[
\text{Pectin content as } \% \text{ calcium pectate} = \frac{\text{Wt. of calcium pectate} \times 500 \times 100}{\text{Ml of filtrate taken} \times \text{Wt. of sample taken}}
\]

d) Estimation of reducing sugar

Sugar content was estimated by standard method (Oser, 1979). 500mg of pulp was taken in 50ml distilled water and boiled, then filtered and the filtrate was diluted upto 100ml. Three Folin-wu tubes were treated as (1) blank tube - distilled water 2ml, (2) 2ml glucose 'C' solution, and (3) 2ml filtrate. In each tube 3ml alkaline copper sulphate solution (40g sodium carbonate, 7.58g tartaric acid and 4.5 g crystalline copper sulphate in 1000ml distilled water) was added, boiled in water bath for 8 minutes. The tubes were cooled and 2 ml of phosphomolybdic acid was added which gave blue color. Then this solution was diluted upto 25ml with distilled water and optical density was determined at 420nm and the total reducing sugar present in pulp was calculated.

e) Estimation of ash

A change in ash contents was estimated following method recommended by A.O.A.C. (1970) and Mungikar (1999). Two gram of oven dry mango pulp was placed in a previously weighed crucible and it was
subjected for heating on hot plate till the sample was sufficiently turned black about 30 minutes. Then it was placed in muffle furnace, pre-heated to 600°C for 2 hours with automatic control. Crucible were transferred directly to desiccators, cooled and weighed immediately. Ash content was calculated as mg/100ml pulp.

f) Estimation of calcium

Calcium content was estimated following A.O.A.C. (1970). An aliquot (25 ml) of the acid solution ash portion was diluted to about 150 ml with distilled water. Few drops of methyl red were added and the mixture was neutralized with ammonia (NH₃) solution till the pink colour changes to yellow. The solution was heated to boiling and the 10 ml ammonium oxalate solution was added. The mixture was allowed to boil for a few minutes. Glacial acetic acid was then added till distinctly pink colour reappeared. The mixture was then kept aside for 12 to 24 hours at room temperature. When the precipitate at calcium oxalate settled down. It was filtered through Whatman filter paper no.42. The precipitate was washed several times with distilled water, to make it free from acid. It was then transferred in a small beaker by piercing a hole in the filter paper and by pouring over it about 15 ml 2N H₂SO₄. This was heated to above 40°C and titrated against 0.01N KMnO₄ solution until the first drop which gives the solution a pink coloration persisting for at least 30 second.

The amount of calcium was calculated using an equation. 1ml of KMNO₄=0.2004 mg of Ca. The percent Ca on DM basis was then calculated on the basis of the amount of sample used for ashing, the volume to which acid solution of ash was diluted and the volume of the aliquot was taken for the precipitation of calcium.

g) Estimation of phosphorus

The estimation of phosphorus was carried out by the method given by Fiske and Row (1925) and recommended by Oser (1979). 0.5ml of acid soluble portion of ash was taken in a test tube. It was diluted to a volume of 10ml with distilled water. Simultaneously blank containing only 10ml
Materials and Methods

distilled water was taken. After that, 1ml molybdate solution (0.25%) was added to each test tube and was mixed, then 0.4ml ANSA (Aminonaphthosulphuric acid) reagent was added and again it was mixed. It was allowed to stand for 5 minutes and optical density (O.D.) was taken at 660mn using colorimeter by setting it to a zero with the blank. The O.D. of standard phosphorus solution was established by preparing a standard graph containing 0 to 1ml standard phosphorus solutions in series of test tubes. Phosphorous content was calculated by using standard graph.

h) Estimation of ascorbic acid

Vitamin C content was estimated by standard titration method. 5 ml of standard solution of standard ascorbic acid (100mg /ml) was pipette out into a conical flask, then 10ml of 0.4 % oxalic acid was taken and it was titrated with dye solution. After that 2gm sample was extracted in 0.4% oxalic acid and volume was made up to 100ml by 0.4% oxalic acid. From that solution 5ml of sample was pipette out into conical flask and titrated with dye solution. End point was pink colour. Finally amount of ascorbic acid in mg / 100ml pulp was estimated by using following formula.

\[
\text{Amount of ascorbic acid mg / 100ml pulp = } 0.5\text{mg} / V_1 \text{ml} \times V_2\text{ml} / 5\text{ml} \times 100\text{ml} / \text{wt. of sample} \times 100.
\]

Where, \( V_1 \text{ml} = \text{volume of Standard Ascorbic acid.} \)
\( V_2\text{ml} = \text{volume of sample’s Ascorbic acid.} \)

C) Control of post-harvest diseases

1) Biological control

Healthy and matured mango fruits were surface sterilized with 0.1 % HgCl₂ and inoculated with seven day-old culture of pathogen using cork wounding method. Then these inoculated fruits were dipped in essential oils emulsions / solutions, plant extract, latex and gums solutions. Untreated fruits were served as control. After that fruits were placed at 30°C at 75- 90 percent relative humidity. Severity of rot was assessed on 8th day of inoculation.
2) **Hot water treatment**

Fresh, healthy and matured mango fruits of Kesar variety were collected from Aurangabad fruit market. Mango fruits were surface sterilized with 0.1 % HgCl$_2$, Pricked to a depth of 2 mm and washed with sterile distilled water. The injured fruits were dipped in fungal spore suspension (5X10$^4$ ml$^{-1}$) for 2min. After inoculation, the fruits were incubated at 25$^0$ C and 90 per cent relative humidity for 24 hours. The inoculated fruits were dipped in hot water at 45, 50 and 55$^0$ C for 5,10 and 15 minutes. Control fruits were dipped in tap water at ambient temperature. After treatment fruits were air dried and incubated for nine days. Severity % was observed on ninth day.

**Statistical analysis**

All the data was statistically analyzed for C.D. following Panse and Sukhatme (1978).