3. MATERIALS AND METHODS:

3.1 Samples collection:

Samples were collected from different regions of Marathwada i.e. Nanded, Aurangabad, Parbhani and Latur districts during 2009 to 2011. Mostly infection was found with leaves and fruits; these infected plant materials were collected for further study.

3.2 Surface sterilization of samples:

The collected infected plant materials were cut aseptically with sterile blades into small pieces and these pieces were soaked in 0.1 % mercuric chloride for 30 seconds then transfer them in 70% ethanol for 1 min as surface sterilizer. After the treatment material was washed 2-3 times with distilled water. Material was soaked in saline for 24 hrs and then crushed it with sterile glass rod in sterile watch glass further this suspension was used for isolation of microorganisms.

3.3 Isolation of pathogenic microorganisms:

The suspensions of selected microorganisms were used for the preparation of dilutions and different dilutions were spread on YDC (Yeast extract, Dextrose, Calcium carbonate) agar plates. All these plates were kept for incubation at 28°C for 3-4 days. After 3-4 days incubation appearance of yellow or white coloured mucoid colonies
indicate the presence of *Xanthomonas sps*. These colonies were further used for biochemical tests (specific for *Xanthomonas sps*) for identification. After confirmation, suspension of that colony was made and streaked on sterile YDC agar plates; incubate for 3-4 days. Using those isolated colonies pure culture was maintained on YDC slants (Collin and Lyne, Microbiological Methods) (Bradbury J.F. 1984, Genus I).

3.4 Biochemical test for *Xanthomonas sps.* :-

3.4.1 *Gram staining* :-

Thin smear of selected colony was prepared by uniformly spreading on a clean grease free slide after that dry the slide and heat fixed it. Firstly that smear was flooded with crystal violet for one minute, after one minute slide was washed with distilled water and further flooded with iodine for one minute then washed with distilled water. Decolourization was carried out by using 95% ethanol and colours flows from the smear. Then safranin was used to flood the smear then washed with distilled water then the slide was blotted with filter paper, dried it in air, and observed under oil immersion objective (Goszozynska *et al.*, 2000, Aneja *et al.*, 2003).

3.4.2 *Motility test* :-

The suspension of *Xanthomonas* was used for motility test on clean coverslip and grease was applied to four corners then cavity slide was put on it. Coverslip was turn to inverting position so that the drop was kept in hanging position as it helped to study the
motility and motility was checked by observing the slide under 40X objective (Aneja et al., 2003).

3.4.3 Catalase test:-

Small part of colony was taken with the help of sterile wire loop and deeped into test tube containing 3% H$_2$O$_2$ (Hydrogen peroxide) solution and observed for bubbles formation (Goszoynska et al., 2000).

3.4.4 KOH solubility test:-

A drop of 3% KOH was placed on a clean grease free slide and a part of single, well isolated colony of *Xanthomonas*, was separated with the help of sterile wire loop and mixed it with that 3% KOH solution. The wire loop was lifted from the solution and observed thread formation. Formation of mucoid thread gave positive result to the test.

3.4.5 Oxidase test:-

N, N, N, N – tetra methyl –P- Phenylene diamine dihydrochloride (oxidase) discs were used for the test. A culture of *Xanthomonas* was put on that oxidase discs and observed it for formation of dark purple colour. If dark purple colour was observed within 30 sec. that indicates strongly positive test, if it gave dark colour within 60sec. that test was weakly positive and if it required more than 60 sec. for observation of dark purple colour that indicated negative test (Goszoynska et al., 2000).
3.4.6 Nitrate reduction test:

Medium used for nitrate reduction test is as, KNO₃ - 1gm, Peptone - 5 gm, Yeast Extract - 3 gm, Agar - 3 gm, Distilled water – 1000ml.

Autoclaved at 121°C for 20 min under 15 lbs pressure. This medium was used to test the ability for reduction of nitrate to nitrite. For that test stabs were prepared and inoculated with culture of Xanthomonas and incubated at 28°C. Bubble formation was observed and it indicated positive result (Goszynska et al., 2000).

3.4.7 Growth on Asparagine medium:

Medium used for this test was as, Asparagine – 0.5 gm, K₂HPO₄ -- 0.1 gm, MgSO₄.7H₂O – 0.2 gm, KNO₃ – 0.5 gm, CaCl₂ - 0.1 gm, NaCl – 0.1 gm, Agar-agar – 15 gm, Distilled Water – 1000 ml and pH – 7.

The Asparagine medium was autoclaved at 121°C for 15 min. and further used for growth of bacteria. This test was used as diagnostic test for Xanthomonas as they are unable to use asparagine as a carbon and nitrogen source. Thus the isolates which couldn’t grow on medium were considered for further tests.

3.4.8 Salt tolerance:

For the test of salt tolerance, concentration of NaCl as 1-5 % was used. Thus, five nutrient broth tubes containing different concentration of NaCl were prepared. Xanthomonas was inoculated into each tube and incubated for 1-3 days and at 30°C.
Turbidity as a growth in the tube indicated as positive tests and no turbidity indicated as negative tests.

3.4.9 Hydrogen sulphide production:

Hydrogen sulphide production medium contained \( \text{NH}_4\text{H}_2\text{PO}_4 \)-0.5 gm, \( \text{K}_2\text{HPO}_4 \)-0.5 gm, \( \text{MgSO}_4\cdot7\text{H}_2\text{O} \)-0.2 gm, \( \text{NaCl} \)-5 gm, yeast extract – 5 gm, Peptone – 0.5 gm was prepared, and 5ml of the medium was transferred in each test tube. All test tubes were sterilized at 121°C for 15 min. Filter paper strips were prepared as 1 x 10cm in size, then filter strips were dipped in 5% lead acetate solution, air dry and autoclaved those strips. The isolates of \textit{Xanthomonas} were inoculated into the medium and lead acetate strips suspended over the medium, incubated for up to 14 days. The presence of black colour on the area of that strips was recorded and indicated as positive test for \( \text{H}_2\text{S} \) production (Goszozynska \textit{et al.}, 2000).

3.4.10 Gelatine liquefaction:

For Gelatine liquefaction test, Gelatin medium (Beef extract- 3gm, Peptone-5 gm and Gelatine- 120gm, distilled water 1000 ml) was prepared, autoclaved at 121°C for 15 minutes and 5 ml of medium was transferred into each test tube, maintained in the stab form. Each isolated strains were inoculated into stab of gelatin and then incubated for 3, 5, 7 up to 21 days. Tubes were kept at 5°C for 15 minutes before liquefaction. Liquefaction of gelatine was seen by tilting all respective tubes (Goszozynska \textit{et al.}, 2000).
3.4.11 Urease production:-

Urease production test for identification of *Xanthomonas*, was evaluated in the medium containing NH₄H₂PO₄-0.5 gm, NaCl-0.5 gm, Yeast extract-1.0gm, Cresol red -0.016gm, Distilled water-800ml, 10% filter-sterilized aqueous urea solution-200ml (separately sterilized), transferred 5ml of media per test tubes and inoculated with isolates of *Xanthomonas*, incubated for 7 days, and then observed for colour changes to dark pink indicated positive reaction for this test (Goszozynska et al., 2000).

3.4.12 Aesculin hydrolysis:-

Isolates were identified by Aesculin hydrolysis test. The medium contain Peptone-10 gm, Aesculin-1.0gm, Ferric Citrate-0.5 gm, Agar-15 gm in 1000 ml Distilled water that medium was equally distributed into each test tube and thus slants of Aesculin were prepared. Each sample of isolates was streaked on slants and incubated for 24-48 hrs. then observed for black colour formation and uninoculated slant was used as a control. Black colour formation indicated presence of β-glycosidase activity (Goszozynska et al., 2000).

3.4.13 Milk proteolysis:-

In milk proteolysis, the degradation of the casein by producing proteolytic exo-enzymes was tested by growing the isolates on milk agar plates. Milk agar medium containing Skim milk powder - 100gm, Peptone - 5 gm, Agar - 15 gm in 1000 ml Distilled water with pH 7.2 was prepared and autoclaved at 121°C for 15minutes.
Observation of clear zone around the colonies of the isolates was recorded as positive test for casein hydrolysis and absence of clear zone indicates negative test (Aneja et al., 1996).

3.4.14 **Indole production**:-

Indole production medium containing Tryptone – 10 gm, Tryptophan - 1.0 gm, Distilled water-1000 ml was prepared and autoclaved at 121°C for 15 minutes. Equal volume of medium was distributed in each test tube. Each isolate was inoculated into the test tube containing medium and incubated at 28°C for 2-5 days. After incubation 0.5 ml Kovac’s reagent (P-dimethylaminobenaldehyde-5 gm, Amyl alcohol-75 ml and HCl-25 ml) was added, formation of cherry red colour on the upper side of the medium indicates positive test and other colour or no colour formation indicated as negative test (Aneja et al., 2003).

3.4.15 **Tween 80 lipolysis**:-

Tween 80 lipolysis medium containing Peptone-10 gm, NaCl – 5 gm, CaCl₂·2H₂O - 0.1 gm, Agar - 15 gm was prepared, mixed it, heated to dissolve, autoclaved at 121°C for 15 minutes, 10ml Tween-80 which was separately autoclaved, then added into the medium, mixed and poured it in plates then the isolates were streaked on plates and incubated for up to 7 days at 37°C after incubation opaque zone were developed around colonies of bacteria, that produced the enzyme esterase and was recorded as positive test, absence of zone around the colony indicates negative test (Goszczynska et al., 2000).
3.4.16 Acetoin production:-

For Acetoin production, medium required was as yeast salts broth and glucose containing NH₄H₂PO₄-0.5 gm, K₂HPO₄-0.5 gm, MgSO₄.7H₂O-0.2 gm, NaCl-5.0 gm, Yeast extracts -5.0 gm, Glucose -5.0 gm. The isolates inoculated in medium of yeast salts broth and glucose with organism, incubated on a rotary shaker for 5 days, transferred 1ml medium to test tubes after 2 to 5 days. After incubation 0.6 ml 5% α- napthol was added and shaked for five seconds, and then 0.2 ml 40% KOH was added and again shaked vigorously, set aside and observed after 30 min., 2 hrs and 4 hrs, appearance of a crimson to ruby colour at the top or throughout the mixture within 2 hrs indicated positive reaction and appearance of colour after 4 hrs indicated negative reaction (Goszczynska et al., 2000).

3.4.17 Starch hydrolysis:-

Starch hydrolysis test is used to study the ability of isolates to hydrolyse starch and detected by using the medium containing 0.2% soluble starch in nutrient agar. The medium was prepared, autoclaved at 121°C for 15 minutes, cooled and isolates of Xanthomonas was streaked on the plates, and incubated at 28°C for 2-7 days. After incubation, the plates were flooded with Gram’s iodine, clear zone around growth or colonies indicated starch hydrolysis and gave positive result.

3.5 Production of pectinolytic enzymes:-
Isolated Xanthomonas strains were used for production of pectinolytic enzymes. The media used for production of pectinolytic enzymes contained

a) Pectin - 5 gm/200ml,  b) KH$_2$PO$_4$ - 1.6 gm, Na$_2$HPO$_4$ - 1.6 gm, MgSO$_4$.7H$_2$O - 0.2 gm, CaCl$_2$.2H$_2$O -0.1 gm, Yeast extract - 5 gm, total contents in 800 ml distilled water with pH 7 was prepared and autoclaved at 121°C for 20 min. under 15 lbs pressure. After sterilisation of media, the suspension of selected strain was inoculated into that media. The flasks containing media were kept for surface fermentation for the production of pectinolytic enzymes.

Thus, various parameters or factors affecting the enzyme production i.e. optimization of cultural parameters like time, temperature, pH, carbon and nitrogen sources were studied further (Starr and Nasuno, 1966).

3.6 Optimization of cultural parameters for pectinolytic enzyme production:

During optimization, the effect of various parameters like time, temperature, pH, carbon and nitrogen sources was checked.

3.6.1 Optimum time for production of pectinolytic enzymes:

The pectinolytic enzyme production media was used for this study. Fifty ml of the medium was taken in each 100 ml Erlenmeyer flask and all flasks were autoclaved at 15 lbs for 20 min. The medium was inoculated with $1 \times 10^7$ cells/ml and mixed thoroughly. These flasks were subjected for different incubation period varied from 1-6 days at 30°C. Culture filtrate was collected every day and kept in refrigerator by adding 1-2 ml of toluene.
3.6.2 Optimum temperature for production of pectinolytic enzymes:

Flasks (100 ml) containing pectinolytic enzyme production media (50 ml) were autoclaved at 15 lbs for 20 min and inoculated with *Xanthomonas* culture \( (1 \times 10^7 \text{ cells/ml}) \). These flasks were incubated for 48 hrs at temperature range from \( 25^\circ \text{C} \) to \( 45^\circ \text{C} \) in surface fermentation (Jayani *et al.*, 2010).

3.6.3 Optimum pH for production of pectinolytic enzymes:

By keeping all other conditions constant the pH of the medium was adjusted in the range of 6.5, 7, 7.5, 8 and 8.5 by using 1N HCl or 1N NaOH in the 100ml flasks containing 50ml of pectinolytic enzyme production medium. All flasks were autoclaved at 15 lbs. for 20 min then after cooling the media \( 1 \times 10^7 \text{ cells/ml } \) of *Xanthomonas* cultures was inoculated in each flask and were kept for incubation at \( 30^\circ \text{C} \) for 48 hrs in static condition.

3.6.4 Effect of carbon on production of pectinolytic enzymes:

To study the effect of different carbon sources on enzyme production, time, temperature and pH of the medium were kept constant, 100 ml flasks containing 50 ml of pectinolytic enzyme production medium with different carbon sources such as pectin, glucose, sucrose, starch and maltose were used at a concentration of 0.5% w/v. These flasks were first autoclaved at 15 lbs. for 20 min and then inoculated with same *Xanthomonas* culture. Flasks were kept for incubation at \( 30^\circ \text{C} \) for 48 hrs.

3.6.5 Effect of nitrogen on production of pectinolytic enzymes:

All 100 ml flasks containing 50 ml of pectinolytic enzyme production medium were prepared with different nitrogen sources such as yeast extract, ammonium nitrate,
ammonium sulphate, potassium nitrate and peptone at a concentration of 0.5% w/v by keeping all other time, temperature, pH and carbon sources conditions constant. All these flasks were autoclaved at 15 lbs. for 20 min. Same *Xanthomonas* culture was inoculated in each flask then all flasks were kept for surface fermentation at 30°C for 48 hrs (Jayani *et al.*, 2010).

### 3.7 Extraction of pectinolytic enzymes:

All incubated flasks were taken for filtration, the culture filtrate was collected, centrifuged at 6000 rpm for 15 min. Supernatant was collected and cell pellets were discarded. That supernatant was filtered through Whatman no. 1 filter paper. This filtrate was further precipitated by drop wise addition of cold acetone with stirring; the precipitate was collected by centrifugation and used as a crude pectinolytic enzyme solution during the course of study (Weber and Parola, 1984).

### 3.8 Assays for pectinolytic enzyme:

Assay of different types of pectinolytic enzymes was performed by using different types of methods. The PG activity was determined by the method described by Gewali *et al.*, (2007). In this method, the reaction mixture contained 2 ml of 1% polygalacturonic acid, 0.2 M phosphate buffer (pH 6.5) and 1 ml of enzyme. Absorbance of the product formed was measured at 240 nm. The activity of Poly Methyl Galacturonase (PMG) was assayed by the method described by Mahadevan and Sridhar, (1986). In this method, the reaction mixture contained 2 ml of 1% pectin, 0.2 M phosphate buffer (pH 6.5) and 1ml of enzyme. Absorbance of the product formed was measured at 240 nm. Pectin Lyase (PnL) activity was determined by the method described by Ramanujam *et al.*, (2008). In this assay, the reaction mixture contained 2 ml of 1% pectin, 0.2 M phosphate buffer (pH 4.5) and 1ml of enzyme. Absorbance of product formed was measured at 240 nm. The
Materials and Methods

Activity of Pectate Lyases was determined by the method described by Vivani et al., (2010), Visser and Voragen, (1996). In this method, the reaction mixture contained 2 ml of 1% polygalacturonic acid, 0.2 M phosphate buffer (pH 4.5) and 1 ml of enzyme. Absorbance of product formed was measured at 240 nm. One unit of enzymatic activity was defined as the amount of enzyme, which released 1 μmol of reducing sugar per minute.

3.9 SDS-PAGE

SDS-PAGE was performed to check the purity and determine molecular weight of pectinolytic enzymes by using standard protein marker. The electrophoresis was carried out by 12.5% gel as per the method of Laemmli (1970). The protein bands were visualized on gel by staining it with Coomassie brilliant blue.

3.10 Media for Production of xanthan gum:

D-Glucose -20 gm, Yeast extract -3 gm, K₂HPO₄ -2 gm, MgSO₄·7H₂O -0.1 gm, Distilled water -1000 ml. Culture was prepared by transferring cells of each variant from a slant to 100 ml Erlenmeyer flask containing 50 ml broth.

3.11 Optimization of cultural parameters for xanthan gum and biomass production:

3.11.1 Effect of incubation time on xanthan gum and biomass production:

The optimal time for xanthan production was determined by incubating the culture at different time intervals as 1st day, 2nd day, 3rd day, 4th day and 5th day. The culture was
3.11.2 Effect of temperature on xanthan gum and biomass production:

To determine the optimal temperature for xanthan production, the culture was incubated at different temperature ranges from 25°C, 30°C, 35°C, 40°C, 45°C and maintained in xanthan production broth. The sample was collected after 24hr interval. The production of xanthan and biomass were determined (Mohan and Babitha 2010).

3.11.3 Effect of pH on xanthan gum and biomass production:

To determine the optimal pH for xanthan production, the culture was incubated at different pH ranges from 6, 6.5, 7, 7.5, 8 and 8.5 and maintained in xanthan production broth. Each sample was collected after specific time interval. The effect of different pH on production of xanthan and biomass were determined (Mohan and Babitha, 2010).

3.11.4 Effect of carbon source on xanthan gum and biomass production:

Different carbon sources such as starch, sucrose, glucose, lactose, and maltose were used to determine the yield of xanthan. Each sample was drawn at every 24 hrs interval for analysis of xanthan and biomass production (Kumara et al., 2012).

3.11.5 Effect of nitrogen source on xanthan gum and biomass production:

Different nitrogen sources such as peptone, yeast extract, Ammonium sulphate, Ammonium nitrate, Pottasium nitrate were used to determine the yield of xanthan. Each sample was drawn at every 24 hrs interval for analysis of xanthan and biomass production (Kumara et al., 2012).
### 3.12 Extraction of biomass:

Biomass was estimated by measuring the dry weight of washed cell mass. After centrifugation, two fractions were formed, supernatant containing xanthan gum, and biomass deposited as a pellet. The biomass pellet was suspended with deionized water for washing and then recentrifuged to reprecipitate the biomass. The biomass deposited at the bottom of tubes was dried in the oven at 60°C for two hours and weighed to get the dry mass per litre culture medium (Kumara et al., 2012).

### 3.13 Extraction of xanthan gum:

The supernatant which previously collected from extraction of biomass was mixed with 2 to 3 volumes of ethanol with continuous shaking to precipitate the xanthan gum. The obtained precipitate was separated by taking the mixture for centrifugation at 6000 rpm for 15 minutes. The collected residue was transferred in micro-centrifuge tube which was previously weighed. This tube was subjected to hot air oven for drying at 60°C for 20 hours. Thus for dry weight of xanthan gum the micro-centrifuge tube was cooled at room temperature then dry weight was taken. The obtained dry weight gave the concentration of xanthan gum by calculating the dry weight of xanthan gum per litre medium (Kumara et al., 2012).

### 3.14 Analytical methods:

#### 3.14.1 FT-IR spectroscopy:
Analysis of functional group of synthesized xanthan gum was performed by using Shimadzu FT-IR spectroscopy. For FT-IR spectra dried powder of xanthan gum was used. This dried powder of xanthan gum was incorporated into KBr i.e. spectroscopic grade and pressed into pellet under reduced pressure. The transmittance mode used during analysis was 4000 to 400 cm\(^{-1}\) (Gilani et al., 2011).

3.14.2 NMR spectroscopy:

For \(^{13}\)C-NMR spectroscopy, the samples were analysed by Bruker AV 300 MHz spectrometer. In this spectrometer, 4 mm magic angle spinning probe head rotor was used. The spectra of samples spinning rate at 8 KHz with 5s recycle time were used. Meanwhile the acquisition time, decoupling field strength and contact time recorded for the spectra was 0.046744 sec, 7.0463 T and 1 msec, respectively.