2. REVIEW OF LITERATURE:

2.1 Taxonomic status:

*Xanthomonas* genus classified under the group of gamma subdivision of *Proteobacteria* and phylum *Gracilicutes*. Further *Xanthomonas* genus is grouped under the family *Xanthomonadaceae* and the order named *Xanthomonadales*. Dowson was the first who proposed the genus named *Xanthomonas* in 1939. *Xanthomonas* strains are identified as phytopathogenic strains as they causes raised and severe lesions to the plant parts. The classification of *Xanthomonas* spps. depending upon the host specificity and infra subspecifically differentiated into the taxa ‘*pathovar*’ as they possess same morphological characteristics. It is reported that strain A, B, C and D are divided on the basis of host as well as regions from where they are isolated. A strain of *Xanthomonas* named as *Xanthomonas axonopodis* pv. *citri* (Asiatic) (Young *et al*., 1996) whereas *Xanthomonas* B strains, C strains and D strains named as *X. axonopodis* pv. *aurantifolii* (South America) (Data Sheets on Quarantine Pests *Xanthomonas axonopodis* pv. *citri*). As DNA-DNA homology observed among the species of *Xanthomonas* it shows diversity in the phytopathogenicity (Vauterin *et al*., 2000).

2.2 Morphological properties:
Xanthomonas strain is identified as Gram-negative, obligate aerobic and slender rods. They are motile bacteria as they have a single polar flagellum. They also differentiated from others by formation of yellow coloured colonies as they produce yellow pigment named as Xanthomonadins. Xanthomonas produces slow growing colonies in culture and they are obligate plant parasites (Bradbury, 1984). Along with the yellow pigments, xanthan gum in the form of extracellular polysaccharide is also secreted by the strains of Xanthomonas at the outer surface which forms mucoid colonies and protect the cells from UV damage as well as included in the interaction of host and pathogen during the period of infection (Bretchneider et al., 1989, Dow et al., 2003).

2.3 Host:

Nearly about 11 families including across 70 genera of 124 monocotyledon plant species are found to be infected with Xanthomonas and it affects to various parts of the plants such as leaves, stems and fruits (Leyns et al., 1984). All citrus cultivars are affected by the Xanthomonas axonopodis pv. citri. Almost hundreds of plant species get affected by this strain of Xanthomonas (Hayward et al., 1991, 1993). Specifically the hosts susceptible for this kind of lesions are grouped under the family Rutaceae. Xanthomonas also infect Solanaceous and Brassicaceous plants (Hayward et al., 1993). Mostly the symptoms caused by all type of Xanthomonas strains are same hence for their separation from each other was carried out with help of host specificity or host range. Bui Thi Ngoc et al., (2009) stated that there are mainly five forms of Xanthomonas citri strain. These are denoted as A, A*, A\textsuperscript{w}, B, C, out of these first three are originated from Asia while remaining last two were isolated from South America.
Stall and Seymour (1983) reported that strain A, A*, A_w have wide range of host as compared with other i.e. B and C strains. A, A*, A_w these strains are identified as Asiatic group and present under the of Xanthomonas citri pv. citri whereas the name Xanthomonas citri pv. aurantifolii referred for the group B and C. Shubert et al., (2002) studied the symptoms of citrus canker as developed on aerial parts of the trees by forming corky lesions.

2.4 Xanthomonas axonopodis pv. citri:

The growth of Xanthomonas axonopodis pv. citri is depend upon the temperature. The minimum temperature required for the growth of Xanthomonas axonopodis pv. citri is 5°C to 9°C and maximum temperature required is 30°C to 39°C (Dye and Lelliot 1974). Time required for their growth is comparatively high than other bacteria (Agrios 2005; Weller and Saettler 1980). The species named Xanthomonas axonopodis pv. citri causes diseases showing symptoms like bacterial blight, necrosis and spots (Leyns et al., 1984). Before the reclassification of Xanthomonas campestris pv. citrumelo, the name known as Xanthomonas campestris pv. citri (Gabriel et al., 1989). Xanthomonas axonopodis pv. citrumelo (Vauterin et al., 1995) and Xanthomonas campestris pv. citrumelo are the synonyms of Xanthomonas axonopodis pv. citri. Throughout the world in citriculture the severe loss was observed due to the pathogenic effect of Xanthomonas axonopodis pv. citri (Mehta and Rosato, 2005) as Xanthomonas campestris pv. citri caused a disease namely citrus canker (Sahi et al., 2007)

2.5 Epidemiology:
It is found that leaves remain susceptible for 6 weeks after initiation of flush. If infection caused after susceptibility it shows the symptoms like severe lesions or wounds due to the thickness of mature tissues. The young trees are more susceptible to the infection caused by *Xanthomonas axonopodis* pv. *citri* than the infection to mature trees. Once the infection caused by the strain of *Xanthomonas axonopodis* pv. *citri* to region of stems of susceptible plants it remain as it is for up to 7 yrs. The bacteria cause disease by growing itself in three phases as pathogenic, epiphytic and survival. The bacteria surviving on organic matter or tools that phase specifically called survival phase, under the favorable conditions the bacteria can grow on host tissue without penetration it known as epiphytic phase and exponential growth of bacteria with tissue penetration called as pathogenic phase (Stall *et al.*, 1983).

### 2.6 Distribution:

Firstly, Citrus canker was discovered in Southeast Asia. Then subsequently the disease was spread throughout Asia as China, Japan, Taiwan, Sri Lanka, and The Philippines. From Asia it spread to Africa, Indian Oceans, South America and USA. Now days, the disease has occurred in islands of the Indian Ocean, in the Middle East and in North America. (Rossetti, 1977, Stall, 1983) (Data Sheets on Quarantine Pests *Xanthomonas axonopodis* pv. *citri*). Specially A strain spread all over Asia including in Afghanistan and Bangladesh. They are also present in China mainly Guangdong, Guangxi, Guizhou, Hubei, Hunan, Jiangsu, Jiangxi, Sichuan and Zhejiang as well as found in Hong Kong. In India, they are specifically observed in the region of Andaman Islands, Andhra Pradesh,
Assam, Haryana, Karnataka, Maharashtra, Punjab and Tamil Nadu. Ibrahim & Bayaa, (1989) have found the A strain of *Xanthomonas* in Iran and Iraq. They are isolated from the region of Japan mainly in Honshu, Kyushu, Ryukyu Archipelago, and Shikoku. As Korea is grouped under the Asiatic region there also A strain were observed. Peninsular and Sabah are the main regions of Malaysia where these bacteria were observed. Along with these regions the strain of A type found in some other regions like Myanmar, Nepal, Oman, Pakistan, Philippines, Saudi Arabia, Singapore, Sri Lanka, Taiwan, Thailand. In South America mainly in Argentina along with A strain, the B strain are also noticed, in the area of Brazil A and C strains are found. In Paraguay the three strains as A, B and C were isolated, in 1985 along with these region east and west of Chaco central, Uruguay it was concluded that A strain and B strain were present (Rossetti, 1977, Stall, 1983).

### 2.7 Pectin and pectinolytic enzymes:

Pectinolytic enzymes maintain ecological balance by causing decomposition and recycling of waste plant materials containing pectic substances (Jayani *et al.*, 2005). Pectic substances are classified into protopectin, pectinic acid, pectin and polygalacturonic acid, depending upon degree of esterification (Gummadi *et al.*, 2007). Protopectin is parent pectin substance, yields pectin or pectinic acid by restricted hydrolysis. Pectic acid composed of galacturonans with negligible amounts of methoxyl groups. Pectinic acid composed of galacturonans with various amounts of methoxyl groups. Pectin contains pectinic acids as the major components (Kiro Mojsov, 2010). Pectin is high molecular weight complex heteropolymers present in middle lamella and
primary cell wall of higher plants. It is made up of D-galacturonic acid residues linked by α-1, 4 linkages (Patil and Choudhari 2010). It is composed of α-D-galacto pyranosyluronic acid (α-D-Galp A) and α-L- rhamnopyranose (α-L-Rhap), α-L-arabinofuranose (α-L-Araf) and B-D-galactopyranose (B-D-Galp) (Yapo, 2011). The production of pectinolytic enzymes and effect of different temperature, pH, carbon and nitrogen sources on their production studied by several Scientists. Pectinolytic enzymes include polygalacturonases (PG), pectinlyases (PL), pectatelyases (PgL), polymethyl galacturonases (PMG); this classification is based on their mode of action. It degrade homogalacturonan and rhamnogalacturonic acid of pectin to convert it into sugar and other useful compounds. They may be used in the pretreatment of waste water from vegetable food processing that contains pectin residues (Pedrolli et al., 2009). Spoilage and decay of the food causes various problems which can be solved by treatment of efficient pectinases. In fruit and vegetable industries, pectinases are used to increase yield and clarification of juice (Sharma et al., 2011).

2.7.1 Polygalacturonases (PG):

Somogyi, 1952 described the method in which incubation of substrate (polygalacturonic acid) with filtrate containing enzyme gives D-galacturonic acid as the final product of reaction. It is reported that testing of PG was done by Biocon method (Wang and Keen, 1970). Detection of polygalacturonase was carried out by Blanco et al., (1994) in which polygalacturonic acid containing solid or liquid media was used. Yoshida et al., (1984), Ross et al., (1993), Hangerman (1986) and Gewali (2007) reported the assay of polygalacturonases. PG enzymes are classified under the class of depolymerising hydrolytic pectinases (Katerlin Belafi Balco
et al., 2006). They have reported that hydrolysis of Pectin or Pectic acid was carried out by Polygalacturonase enzymes.

2.7.2 Polymethylgalacturonases (PMG):


2.7.3 Pectin Lyases (PnL):

Sathyanarayana and Panda (1998) described the method for assay of pectin lyases. Enzyme and substrate mixture was kept for incubation at room temperature for 1 hr and further HCl was added to stop the reaction. Sangeeta et al., (2009) concluded that, due to the end product i.e. 4, 5-unsaturated galacturonosyl residues, pectin lyases are specifically differentiated from other type of pectinases as other pectinases degrade pectin molecule completely. Along with this assay method some other methods like reducing group methods, viscosity reduction methods are reported by Roboz et al., (1952). Albersheim et al., (1966) concluded that thiobarbituric acid (TBA) was useful for detection of unsaturated compound (uronic ester) for quantitative analysis of pectin lyases activity by colorimeter.

2.7.4 Pectate Lyases (PgL):
As per the previous literature it was noted that the activity of pectate lyases was assayed by measuring the increased amount of reducing group with decrease in viscosity (Macmillan, 1964). Whitaker, (1990) reported that pectate lyases activity was checked by formation of 4, 5 double bonds to the position of nonreducing ends of unsaturated products. This is the standard assay method concluded by Collmer et al., (1988). For the assay of pectate lyases, Zucker and Hankin in (1970) used the spectroscopic analysis test. In this assay they have observed the increase in absorbance at 235 nm with increased amount of unsaturated uronides specifically named those uronides as oligo galacturonides.

2.8 Optimization of pectinolytic enzyme production:

2.8.1 Effect of incubation time on pectinolytic enzyme production:

To study the effect of incubation time on pectinolytic enzyme activity Jayani et al., (2010) specifically in case of polygalacturonase, used the range of incubation time as 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs, out of that they concluded that the maximum production was observed at 72 hrs whereas Loera et al., (1999) reported that 73 hrs incubation time is the optimum time for maximum production of polygalacturonase enzymes. PMG also named as pectin hydrolases (Itziar et al., 1998), production was studied by determining the production of reducing sugar (Collmer et al., 1988). Panda et al., (2004) reported in their study that polymethyl galacturonase enzymes synthesized by Aspergillus niger showed maximum production at 144 hr of incubation and they also noticed that the production of PMG enzyme was started after 60 hr. Ramanujam et al., (2008) have reported that the pectin lyase enzyme synthesized by Aspergillus niger showed maximum production at 84 hrs of incubation time.
Pectate Lyase production was observed maximum at 96 hrs of incubation time (Viviani et al., 2010).

2.8.2 Effect of temperature on pectinolytic enzyme production:

Tsuymu (1979) suggested that optimum temperature for production pectate lyase enzymes, synthesized by Erwinia carotovora was 40°C. Jong et al., (1998) concluded that, pectin lyases which are synthesized by Bacillus species showed the maximum production at 40°C, whereas Yadav and Shastri, (2004), reported that the optimum temperature for the production of pectin lyases synthesized by Aspergillus niger is 35°C, Jayani et al., (2010) used the temperature range of 25°C, 30°C, 35°C, 40°C and 45°C for the optimization of polygalacturonase production. For the study of optimization, they used the enzyme polygalacturonase, synthesized by Bacillus sphaericus. They concluded that 30°C was the optimum temperature for the PG production. Several researches suggest the optimum temperature for polymethyl galcturonase synthesized from Bacillus species was about 35°C (Rastegari and Heidari, 2014).

2.8.3 Effect of pH on pectinolytic enzyme production:

Jong et al., (1998) reported that pH 6 was the optimum pH for the production of pectin lyases synthesized by Bacillus species. Soares et al., (1999) concluded that the optimum pH required for the production of polygalacturonases by Bacillus species was almost near to neutral pH. Yadav and Shastri, (2004) studied that the maximum production of pectin lyases synthesized by Aspergillus niger occurred at pH 5.5 among the range used as 5 to 8. The
pectate lyases synthesized by *Penicillium viridicatum* showed maximum production at pH 5.5 as stated by Viviani *et al.*, 2010. Polymethyl galacturonase enzyme isolated from *Bacillus species* showed maximum production was found around neutral pH (Rastegari and Heidari, 2014).

### 2.8.4 Effect of carbon sources on pectinolytic enzyme production:

The composition of the media plays an important role in the study of optimization of pectinolytic enzymes production. Maldonado *et al.*, (1989) reported that the maximum production of polygalacturonase enzyme observed in the media containing pectin as a carbon source. In the study of effect of carbon source on the production of pectinolytic enzymes, it is reported that the maximum production of pectin lyases synthesized by *Bacillus species* was observed in media containing pectin as a carbon source (Kashyap *et al.*, 2000). Pectin was observed as a suitable carbon source for maximum production of pectinolytic enzymes.

### 2.8.5 Effect of nitrogen sources on pectinolytic enzyme production:

Kashyap *et al.*, (2003) resulted that the production of polygalacturonases showed maximum in the media which contain yeast extract as a nitrogen source. Azzaz *et al.*, (2013) reported that the maximum production of pectinolytic enzyme synthesized by *Aspergillus niger* was observed in the media containing yeast extract as nitrogen source. Yeast extract was found to be the most efficient nitrogen source for the production of pectin lyases which are synthesized by *Bacillus sps* (Kashyap *et al.*, 2000).

### 2.9 Xanthan gum:
The concentration of xanthan gum as an exopolysaccharide produced by the strain of *Xanthomonas* play an important role in the pathogenicity of this species (Bretchneider *et al.*, 1989, Dow *et al.*, 2003).

The plant pathogenic bacterium *Xanthomonas campestris*, produced an anionic polysaccharide named xanthan gum, has been widely used in food products (Liu *et al.*, 2012). Xanthan gum consists of a linear 1, 4-linked β-D-glucose backbone as a main chain, substituted on every two unit with a charged tri-saccharide side chain. Other side chain is composed by a D-glucuronic acid unit linked between two D-mannose units (Severine *et al.*, 2012). The gum exhibits many advantages as a thickener, stabilizer, gelling agent and suspending agent, as creams, artificial juices, sauces for salads, meat, chicken or fish, as well as for syrups and coverings for ice creams and desserts as it has high degree of stability and solubility (Sandra *et al.*, 2011). Xanthan gum is combined with galactomannans for the use as a gelling agent due to its weak gel structures (Kiosseoglou *et al.*, 2003).

Xanthan is useful in many industries as its nature as a non-toxic, it is also tested that xanthan does not inhibit growth. Xanthan is more beneficial as it is non-sensitizing and does not cause skin or eye irritation (Aarthy and Vijayakumar, 2011). Xanthan gum was discovered in late 1950s when an American research team, which was conducting extensive search for useful biopolymers (Riadh *et al.*, 2010). Cultural conditions and various compositions of media are considered as the factors affected xanthan gum production. Mainly xanthan gum production is influenced by the factors such as temperature, pH, time, carbon and nitrogen sources (Nasr *et al.*, 2007; Borges *et al.*, 2008; Papagianni *et al.*, 2001). Xanthan gum production or fermentation proceeds up to the recovery. For the recovery of xanthan gum the processes like filtration and centrifugation are specifically used. Purification of
xanthan gum is carried out by precipitation, dewatering and drying of the filtrate (Flores-Candia and Deckwer, 1999).

2.10 Optimization of xanthan gum production:

2.10.1 Effect of incubation time on xanthan gum production:

To study the effect of incubation time on the xanthan gum production several scientists suggested various time intervals such as 24 hrs, 48 hrs, 72 hrs, 96 hrs, 120 hrs. Fermentation time plays an important role in xanthan gum production. The presence of amount of acetyl group and pyruvate depends on the time required for the process of fermentation (Sandford et al., 1977). It is concluded that if the variation occur in these groups it affect the rheology of xanthan gum (Holzwarth and Ogletree, 1979). Shu and Yang, (1990) stated that the xanthan gum production was influenced by the fermentation time. Casas et al., (2000) reported that the percentage of pyruvilation, acetylation and the average molecular weight of extracellular polysaccharide i.e. xanthan gum was found to be increased with increase in fermentation time. Marzieh et al., (2010) demonstrated that strain Xanthomonas campestris showed maximum production of xanthan gum after 96 hrs of fermentation.

2.10.2 Effect of temperature on xanthan gum production:

The study of effect of temperature on xanthan gum production is also significant in this study. The temperature used for xanthan gum production depends on the composition of media used for its production (Shu and Yang (1990, 1991). Borges et al., 2008; Gumus et al., 2010; Psomas et al., 2007; Silva et al., 2009; Kerdsum et al., 2009 and Garcia-Ochoa et al., (1992) reported that the optimum temperature for xanthan gum production was 28°C. The optimum temperature for production of xanthan gum was represented between 25–27°C (Esgalhado et
Thonart et al., (1985) suggested that the optimum temperature for the growth of Xanthomonas strain was 25°C and 30°C temperature was optimum for the production of xanthan gum. At temperature 30°C the strain of Xanthomonas axonopodis pv. vesicatoria showed maximum production of xanthan gum (Mustafa et al., 2011).

2.10.3 Effect of pH on xanthan gum production:

The neutral pH required for optimum growth of strain of Xanthomonas was reported by (Gumus et al., 2010; Kersup et al., 2009; Psomas et al., 2007; Silva et al., 2009). In case of xanthan gum production the optimum pH was considered at slightly below than neural pH. Usually the pH range used by several scientists is between 5 to 8. The xanthan gum production was optimum at pH lower than neutral as they produce acid during the process fermentation (Borges et al., 2008). Mustafa et al., (2011) studied that the neutral pH of the media during fermentation showed maximum production of xanthan gum using strain of Xanthomonas axonopodis pv. vesicatoria.

2.10.4 Effect of carbon sources on xanthan gum production:

For the production of xanthan gum the appropriate concentration of carbon source is required. Higher concentration as well as lower concentration showed adverse effect on the production of xanthan gum (Palaniraj and Jayaraman, 2011). Various researches reported that different composition of carbon sources required, depend upon the desired side chain of xanthan gum (Davidson, 1978; Souw and Demain, 1979; Garcia-Ochoa et al., 1992; Letisse et al., 2001). The various types of carbon sources were studied with the fermentation of wild type Xanthomonas campestris GK6 (Leela and Sharma, 2000). Carbon and nitrogen ratio with high value gives high production of xanthan gum (De Vuyst et al., 1987). Kumara et al.,
(2012) demonstrated that sucrose was the most effective carbon source for maximum production of xanthan gum.

**2.10.5 Effect of nitrogen sources on xanthan gum production:**

The maximum incubation time for the production of xanthan gum was required due to high degree of pyruvilation and acetylation (Casas et al., 2000). The amount of nitrogen source added to the fermenter at the beginning of stationary phase affects the amount and rate of xanthan gum production (Pinches and Pallent, 1986). Different nitrogen sources such as peptone, yeast extract, ammonium sulphate, ammonium nitrate, potassiam nitrate were used to determine the yield of xanthan (Kumara et al., 2012). They reported that yeast extract was the most suitable nitrogen source for the maximum production of xanthan gum as well as biomass which was synthesized by *Xanthomonas campestris* isolated from soil.

**2.11 IR- spectroscopy:**

**2.11.1 FT-IR spectroscopy:**

FT-IR spectroscopy is the sophisticated analytical technique, for the characterization and comparative analysis of xanthan gum which was derived from waste with the standard xanthan gum was carried out by using FT-IR spectroscopy (Kedar J. A., 2014). Detection of similarities and differences in the structure of xanthan gum was analysed by using the technique of FT-IR spectroscopy (Sandra et al., 2011).

**2.12 NMR-spectroscopy:**

**2.12.1 $^{13}$C- NMR spectroscopy:**
Rinaudo et al., 1983, discussed in their work that the analysis of xanthan gum was done by using NMR spectroscopy. Few papers are available on the work of analysis of xanthan gum by using NMR spectroscopy. Use of NMR spectroscopy is due to the ordered and rigid conformation of natural xanthan gum in solution, which is can not be detected by usual high resolution conditions as it produce strong dipolar interaction between proton or carbon nuclei. The analysis of chemical structure of xanthan gum was carried out by using the technique namely $^{13}$C- NMR spectroscopy (Denise et al., 2011).