Antagonistic activity of cellulase enzyme produced by *Trichoderma Viride* against *Xanthomonas Citri*

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**ABSTRACT**

Citrus canker is a bacterial disease which occurs in *citrus* (lemon) species produce by *Xanthomonas* especially *X. citri*. The infection occurs on the lesions of leaves, stems, and fruits of citrus trees, it also occurs in other plants such as orange, grapes. The main goal of this study was to prevent the lemon (*citrus*) disease such as citrus canker by using some cellulase enzymes produces by soil fungi. In the present study, we isolated soil fungi *Trichoderma viride*, which produces cellulase enzyme these have higher antibacterial activities against plant pathogen such as *X. citri*. This enzyme produced by *T. viride* by using fermentation method, quantified by DNAs method and used as a bio-control agent against *X. citri*. They also isolated and characterized *X. citri*. In the last step of the study they have checked antibacterial activity of cellulase enzyme against *X. citri*. The present study concluded that cellulase enzyme produced by *T. viride* shows higher antibacterial activity against *X. citri* when treated it at 100°C.

**Key words:** Antimicrobial activity, Cellulase enzyme, Characterization, Fermentation, Quantification, TLC, *Trichoderma*, Xanthomonas.

**INTRODUCTION**

Lemon has an important value as fruit crop and economically good for farmers. In the 21st century lemon is delectable, juicy, and has great nutritional significance (Khan et al., 1992). Lemon is a member of citrus family and grown in changeable densities in countries with tropical or subtropical climates. It is used as best source of ascorbic acid (vitamin C), sugars, amino acids and other nutrients (Ahmed and Khan, 1999). Citrus canker is one of the most destructive and predominant disease of acid lime in the world as well as India. In the central region of India citrus canker is one of the most predominant disease which occurs in lemons. In the Sehore district of Madhya Pradesh (India) the disease affects most of the crops of lemon. Citrus canker is a bacterial disease that causes lesions on the leaves, stems, and fruit of plants including citrus and other plants like orange, grapes in the *Rutaceae* family. The disease does not affects human, this is plant disease mainly affect the crops of lemon. The symptoms of the disease consist of brown spots on leaves, often with an oily or water-soaked appearance. Disease of lemon is usually spotted on fruit peel and leaf surface but when the atmosphere is very favorable for contamination, the infections spread in whole plant parts. The citrus canker caused by the bacteria *X. Citri*, this is a gram’s negative, rod-shaped bacteria with polar flagella. The bacterium has a genome length of around 5 mega-base pairs. The growth of this bacterium is obligatorily aerobic, and the optimum temperature for growth is 35°C to 39°C (Mehrotra, 1980; Whiteside, et al 1988). They have capability to form a biofilm for connection on the host; the biofilm ensures the virulence and epiphytic survival of *X. citri* earlier to the increase of citrus canker. They have the capability to form a biofilm to makes the connection on the host; the biofilm ensures the virulence and epiphytic survival of *X. citri* earlier to the increase of citrus canker. The effector interacts through host machinery to make transcription used for genes that control plant hormones such as auxin and gibberellin (Pereira et al., 2014). The species of *Trichoderma* is a hyper-parasitic pathogenic fungi which have highly efficient antagonist activity (Barnett and Binder, 1973; Durrell et al., 1968). *Trichoderma* is commonly present in the soil, especially waterlogged soil, dung and decaying plant materials, and familiar because of its antagonistic action and involvement in food processing and biodegradation.

To get rid of citrus canker disease they have used *T. viride* as a bio-control agent and produced cellulase enzyme by *T. viride*, it is a green mold which is commonly fungi found in soil. It can produce a variety of enzymes, including cellulase and chitinase which can degrade cellulose and chitin respectively. Cellulase enzyme has become the important biocatalysts due to their complex nature and large spread industrial applications. Cellulase is composed of separately folding, structurally and functionally discrete units called domains or modules, production cellulase module (Henriass et al., 1998). Cellulase enzyme is inducible enzymes synthesized by a large diversity of

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After morphological characterization (Kubicek et al., 1993; Sang and Koo, 2001), the constituents of selective medium in pure culture (SDA media) appeared on solid medium in 5 to 6 days, a single colony was picked up and maintained in pure culture condition. This formation is modified in support of working on an insoluble substrate, and it allows the enzyme to diffuse two-dimensionally on a surface in a caterpillar-like fashion. However, there are also cellulosomes (mostly endoglucanases) that need cellulose binding domains. The enzyme is categorized as an endoglucanases, which internally cleaves β-1, 4-glycosidic bonds in cellulose chains facilitating further degradation of the polymer. Particular group in the similar family as T. maritima produce cellulase with diverse structures (Cheng et al., 2011). Cellulase enzymes hydrolyze the substrate carboxymethyl cellulose, required of the substrate in the active site induces a change in conformation which allows degradation of the molecule. The method of cellulase enzyme degradation by aerobic bacteria is similar to that of aerobic fungi, it is clear that anaerobic bacteria operate on a different system (Carvalho et al., 2003). In the present study, an antagonistic strain of T. viride capable of controlling X. citri was isolated from pathogen Citrus canker (Lemon disease) and its behavior was studied under in vitro condition.

**MATERIALS AND METHODS**

**Sample collection**: Soil samples were collected from rhizospheres of different crop fields of different locations of nearby regions of Sehore district (M.P.) India in the month of June. At each site, 2 samples were taken randomly. The samples were transported in sealed aluminum foil to the laboratory, stones were removed from the soil samples and the soils were homogenized through a 2mm sieve. The samples were stored in the dark bottles for further analysis.

**Isolation and identification of Trichoderma species**: Prepare selective media for Trichoderma spp. with adding following constituents such as MgSO 4, 7H 2 O-0.2g, K 2 HPO 4 -0.9g, NaNO 3 -1g, KCl- 0.15g, dextrose- 3.0g, rose bengal-3.0g, agar-20.0g, distilled water- 1000ml, pH- 6.5 (Elad et al., 1980), and then after sterilized media and glassware’s at 121°C for 15 min. at 15lbs pressure. Serial dilution method was used, for this purpose the 10^6 dilution of each soil sample were prepared, after that 500μl sample of 10^6 dilution pour and spread on Trichoderma selective medium. The Petri dishes were incubated for 7 to 8 days at 28°C. Colonies appeared on solid medium in 5 to 6 days, a single colony was picked up and maintained in pure culture (SDA media) for further study.

**Isolation and preservation of the canker disease sample**: Isolation of X. axonopus pv. citri was done in YDC media (Scortichini et al., 2001), the constituents of selective medium of X. axonopus pv. citri were yeast extract 5.0g, CaCO 3, 10.0g, dextrose 10.0g, agar-agar 8.5g and 500ml distilled water. It was lightly heated until dissolved and pH was maintained 6.4±0.2 and autoclaved at 121°C, 15 lbs for 15 minutes. Cut the infected parts of leaves and were transferred onto the YDC agar medium and incubated at 28°C±1 for two to three days after incubation, a single bacterial colony was picked up and isolate pure culture (NAM media) for further study.

**Biochemical Characterization Tests of Xanthomonas axonopus pv. Citri Gram staining**: A thin smear of pure bacterial culture was made and dried and heat fixed. Then smear was covered with crystal violet (primary stain) for 30 to 60 seconds. The slide was washed with distilled water for a few seconds. Then smear was covered with gram’s iodine solution for 30 second and then washed, with 95% ethyl alcohol drop. The slides were then washed with distilled water. Further saffranin (counter stain) was applied to smear for 30 second and again slide washed with distilled water the slides were dried and observed under 100X microscope along with one drop mineral oil to examine shape, size, and arrangement and staining reaction of bacterial isolates (Bradbury, 1970).

**Biochemical tests** After morphological characterization they were performed some biochemical test for species level identification like catalase, urease, casein hydrolysis, starch hydrolysis or amylase, gelatin liquefaction, KOH test, fluorescent pigmentation.

**Preparation of inoculums for cellulase enzyme production**: After the completion of biochemical tests and when the species confirmed. After that, the cellulase enzyme
was produced by using the fermentation method, in this process the inoculums of *T. viride* was used. Inoculums were prepared from those culture plates which give a positive test for cellulose degradation. Take two conical flasks and add 50ml Sabouraud dextrose agar (SDA) broth media in each flask then mixed the fungal culture taken from a plate which gives a positive test for cellulose and incubated for five to seven days at 28 to 30°C.

**Batch fermentation method of cellulase enzyme production by** *Trichoderma viride*: Batch fermentation was carried out in 200ml conical flask containing 50ml of fermentation medium. The composition of the medium contained the following g/liter of distilled water. NaNO₃-2.0g, K₂HPO₄-1.0g, MgSO₄. 7H₂O-0.5g, KCl-0.5g, carboxymethyl cellulose (CMC)-5.0g, peptone-2.0g. The medium was sterilized by autoclaving at 121°C for 15 min. in each conical flask 5ml of *X. viride* inoculums was inoculated. The fermentation medium was incubated in fermenting shaker (120 rpm) at normal room temperature for 10 to 15 days (Mrudula and Murugammal, 2011).

**Extraction of cellulase enzyme from fermented media by downstream process:** There are two types of downstream processing filtration and centrifugation. Filtration method used for filtration the fermented culture by cellulase filter paper then again whatman filter paper. Then collect the filtrates and discard the mycelium, then heated at 100°C for 20 to 30min and cooled at room temperature and added equal volume of 20% TCA (Trichloroacetic acid) than kept the sample tube in deep freezer for one hours at -20 °C temperature to precipitate the enzyme/protein.

**Quantitative analysis of cellulase enzyme by Folin Lowry method:** It is protein/enzyme was estimated by the protocol of Folin Lowry method. For that they had taken stock concentration 3mg/3ml and sample was used in (600μl) mid value with Lowry samples, reagent C and Folin-Ciocaltu reagent (FCR) was also used (Patil et al., 2015). Then OD was taken at 660nm in spectrophotometer (Table 1).

**Qualitative analysis of cellulase enzyme by TLC:** To investigate the final products released from the hydrolyzed cellulase, the hydrolysis products obtained from the substrate were analyzed by thin-layer chromatography (TLC) as described by Liu, 2013 (Liu D et al., 2013). A 20μl volume of the sample was loaded onto a silica gel G and separated by a solvent system consisting of chloroform- methanol-distilled water (9:6.5:1.5, v/v) and observe at under UV light.

**Estimation of produced cellulase enzyme by DNSA method:** In the first step prepared a glucose stock solution-3mg of glucose was dissolved in 3ml distilled water (w/v) and in the second step prepared DNSA reagents- for the making of the reagent added 4g NaOH in 30ml distilled water mixed properly after that added 1.5g dinitrosalicylic acid in 30ml NaOH solution and in the last step they prepared 40% potassium sodium tartrate solution by adding 40g potassium sodium tartrate in 100 ml of distilled water (Kondo ., et al 1994). After that, they take eight test tubes and label them as blank and 1 to 7. Make dilutions of glucose standards with different concentrations of 20, 40, 60, 80 and 100μl and sample was 60μl (mid volume) per 100μl by transferred respective quantity of glucose from the standard glucose solution (1mg/ml) and adjust it to a total volume of 100μl by adding distilled water and then added 1ml of DNSA reagent into all the eight test tubes and keep it in boiling water bath for 15 minutes. After boiling add 1ml of 40 % potassium sodium tartrate solution and take OD at 540 nm in spectrophotometer (Table 2).

**Antibacterial activity of cellulase enzyme against *X. citri* by disk diffusion method:** The antibacterial activity was performed using disc diffusion method. For this purpose they treated cellulase enzymes with different temperatures. We take over night grown *X. citri* culture in liquid broth then bacterial culture was spread on Mueller-Hinton Agar Media (MHA media) plates and take four test tubes and poured 1-1ml of cellulase enzyme in each test tube and heated at different temperatures (25°C, 50 °C, 75 °C and 100 °C) for 5

### Table 1: Procedure of proteins estimation by Lowery reagent Method.

| Sample/Standard | DW | Solution C | FCR/Solution D |
|-----------------|----|------------|----------------|
| 20μl            | 80μl | 5ml       | 500 μl         |
| 40μl            | 60μl | 5ml       | 500 μl         |
| 60μl            | 40μl | 5ml       | 500 μl         |
| 80μl            | 20μl | 5ml       | 500 μl         |
| 100μl           | 00μl | 5ml       | 500 μl         |

| Sample/Standard | DW | DNA | Potassium sodium tartrate |
|-----------------|----|-----|---------------------------|
| 20μl            | 80μl | 1 ml | 1 ml                       |
| 40μl            | 60μl | 1 ml | 1 ml                       |
| 60μl            | 40μl | 1 ml | 1 ml                       |
| 80μl            | 20μl | 1 ml | 1 ml                       |
| 100μl           | 00μl | 1 ml | 1 ml                       |
RESULTS AND DISCUSSION

Morphometric characterization of Trichoderma on agar plate and microscopy: In this study, two isolates of Trichoderma were isolated from different soil samples on the basis of morphological characteristics. Green conidia forming fungal bodies were selected, for the identification of Trichoderma fungal staining was performed, in the fungal staining started from taking a clean glass slide after that fungus culture transfer on it and stain with lactophenol and cotton blue, the slide observed under microscope (Fig. 1). Study of morphological characteristics is one of the conventional methods to identify Trichoderma species and it remains as a potential method to identify Trichoderma up to species level (Samuels et al., 2002).

Determination of spore and mycelia size by ocular micrometry: Cell morphology such as length, width (μm) of spore and size of mycelium of T. viride are given in Table 3 and Table 4. Trichoderma grown in the SDA plates at 28°C showed the same growth pattern with different colony characteristics. Size of spore and mycelia were useful in the identification of possible T. species. The color of the colonies varied from light green to dark green. Each T. viride isolate having similar colony morphology, which most probably, belong to the same species (Fig. 2).

Biochemical characterization of T. viride: For the identification of Trichoderma viride various test were used such as cellulase production, chitin production and pectin production. The results of biochemical tests are summarized in Table 5.

Results of biochemical characterization of Xanthomonas axonopodis pv. Citri: The isolated bacteria were identified based on physiology, morphology and biochemical characterization (Fig.3). For the identification of Xanthomonas Citri various tests were used such as catalase, urease, casein hydrolysis, starch hydrolysis or amylase, gelatin liquefaction, KOH test, fluorescent pigmentation test. The results of biochemical test of Xanthomonas Citri given in Table 6.

Production of cellulase enzyme by Trichoderma viride by Batch fermentation: Fermentation was done by Czapek-mineral salt broth media inoculated in conical flask and add inoculums after that incubated in shaking incubator at 28±2°C temperature for 7 to 10.

Extraction of cellulase enzyme from fermented media by downstream process: Extraction was done by Tri chloric acid 20% (TCA) with the help of fermented media. After extraction we performed some qualitative and quantitative test.

Qualitative analysis of cellulase enzyme by TLC: The produced cellulase enzyme characterizes by TLC Chromatogram, TLC slide observed under UV light, band (spot) observed and measured after that calculates the Rf value. The TLC findings are in agreement with other reports. The production of cellulase enzyme also depends upon the type of microorganisms and strains and on their age. The maximum cellulase enzyme was observed in the stationary phase of PSM and other species of Trichoderma. The cellulase enzyme substances were detected by means of thin layer chromatography methods. Qualitative analysis had done by TLC (thin layer chromatography) using chloroform.

| Spices name | Colony size in petri plate(mm) |
|-------------|--------------------------------|
| T. viride   | 5.75 mm                        |
| T. viride   | 5.5 mm                         |

| Spices name | Size of Conidia in μm | Size of mycelia in μm |
|-------------|-----------------------|-----------------------|
| T. viride   | 3.428 μm              | 17 μm                 |
| T. viride   | 4.571 μm              | 26 μm                 |
| T. viride   | 5.714 μm              | 40 μm                 |
| T. viride   | 3.428 μm              | 36 μm                 |
| T. viride   | 4.571 μm              | 32 μm                 |

| Spices name | Cellulase | Chitin | Pectin |
|-------------|-----------|--------|--------|
| T. viride   | +ve       | +ve    | +ve    |
| T. viride   | +ve       | +ve    | +ve    |

| Biochemical tests | Observation |
|-------------------|-------------|
| Gram staining     | -ve         |
| Catalase          | +ve         |
| Urease            | -ve         |
| Casein hydrolysis | +ve         |
| Starch hydrolysis or amylase | +ve |
| Gelatin liquefaction | +ve |
| KOH test          | +ve         |
| Fluorescent pigmentation | -ve |

| Temperature | Zone of inhibition(mm) |
|------------|------------------------|
| 25°C       | 11 mm                  |
| 50°C       | 12 mm                  |
| 75°C       | 14 mm                  |
| 100°C      | 18 mm                  |
methanol and distilled water (9:6.5:1.5) solvent system. \textit{Rf} value of cellulase enzyme 1.55 and 1.62 were recorded (Fig. 4).

**Quantitative analysis of cellulase enzyme by Lowry method:** Quantitative analysis was carried out by using Lowry method, in this regards we prepared BSA standard solution (1mg/ml) (Fig. 5) and calculate the unknown concentration of cellulase enzyme. The results of cellulase enzyme concentration were 62.78 \(\mu\)g/ml and 86.28 \(\mu\)g/ml (Fig. 6).

**Estimation of produced cellulase enzyme by DNSA method:** Enzyme estimation was carried out by using DNSA method. In this method standard glucose solution was prepared (1mg/1ml) after that take different concentration for plotting standard curve. The concentration of cellulase enzyme calculated with the reference of standard curve (Fig. 7) is treated with DNSA method. The quantified concentrations of cellulase enzyme are 55.76 \(\mu\)g/ml and 89.63 \(\mu\)g/ml (Fig. 8).

**Antibacterial activity of cellulase enzyme against \textit{X. citri} by disk diffusion method:** Cellulase enzyme showed higher
Fig 5: Standard curve of BSA.

Fig 6: Results of concentration of cellulase enzyme in μg/ml.

Fig 7: Standard curve of glucose.

Fig 8: Concentration of cellulase enzyme.
antibacterial property against \( X. \text{citri} \) where the inhibition zones were very good (Fig. 9). When the concentration of cellulase enzyme was same but treated the enzyme at various temperatures such as 25°C, 50°C, 75°C and 100°C, the antibacterial activity increased (Table 7).

**CONCLUSION**

In the present study they isolate \textit{Trichoderma viride} from different soil samples which were collected from different agricultural field, \textit{T. viride} shows diverse morphology characteristics and occurred in a wide range of pH and moisture conditions of soil. \textit{T. viride} is capable to produce the cellulase enzyme, this enzyme try to use as a bio-control agent. Cellulase enzyme shows strong inhibitory effect against \( X. \text{citri} \). The results suggest that the secondary metabolites of \textit{T. viride} such as cellulase enzyme give strong antibacterial activity against plants pathogenic bacteria which are cause disease in lemon plants. Present study proved that cellulase enzyme has antibacterial activity against \( X. \text{citri} \). The results of the study indicate that the \textit{T. viride} produced some extracellular substances which have strong antibacterial potential to plant pathogenic bacteria.

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