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Screening of Microorganisms for Chitinase Production and their Utility as Biocontrol Agent against Fusarium Oxysporum

Binumol M[1], Dhiva S [2]
[1]*Post Graduate and Research Department of Botany, Sree Narayana College, Nattika, 680566*
[2]*Department of Microbiology, Sree Narayana College, Alathur, 678682*

Abstract: ***Fusarium wilt of tomato caused by the fungus Fusarium oxysporum f. sp. lycopersici causes great loss in warm climates and sandy soils of temperate regions. Increased use of chemicals, cause several negative effects, like development of resistance to pathogen and increasing the chemical toxicity.***
Chitin is widely found among plants, microorganisms and animals. The chitinase can cleave chitin to yield N-acetylglucosamine, which can be used as a carbon source for microbial growth.
The potentiality of certain microbes to produce chitinase, has been exploited as biocontrol agent against certain fungal pathogens. This work is aimed at isolating potential chitinase producing bacteria which could grow at a temperature of 25 to 30 ^{0} C. Soil Samples from three estuaries near Tellicherry, Dharmadam and Mahe were collected.Screening of chitinase producing bacteria was done by Selective media Chitin agar.
Two isolates of serratia and Pseudomonas were found to have the potentiality of chitinase production. It was found that serratia isolate had the best chitinase activity of 42 mU/ml , when grown in Enzyme production Media having a 20 %(w/v) colloidal chitin as substrate, at 30 ^{0} C with the agitation rate of 150 rpm for 3 days.
Keywords: Fusarium wilt, chitinase,Biocontrol

I. INTRODUCTION

Fusarium wilt of tomato caused by the fungus Fusarium oxysporum f. sp. lycopersici causes great loss in warm climates and sandy soils of temperate regions. Several chemical fungicides such as Bavistin etc. are used to suppress the disease but these chemicals have a negative impact on human health and are hazardous to the environment.
A better alternative to chemicals are the soil microbes such as Trichoderma, Penicillium, etc. residing in the rhizosphere of crop plants that have the ability to suppress the pathogens (Hyakumachi et al., 1994) and stimulate plant growth by the production of phytohormones (Hasan, 2002) and/or degradation of complex substrates (Altmore et al., 1999).
Antagonistic nature of T. virens and Aspergillus against Phytophthora capsici causing foot root disease of black pepper has been reported (Noveriza et al., 2004). Biological suppression of plant disease has been promoted as a means to achieve improved, sustainable crop production systems that are less reliant on chemical inputs (1).
Successful biological control systems commonly employ naturally occurring, antagonistic microorganisms that are able to reduce the activities of plant pathogens.
Such antagonists (or biocontrol agents) can compete with pathogens for nutrients, inhibit pathogen growth by secreting antibiotics, or reduce pathogen populations through parasitism. In addition, some of these microorganisms induce resistance in host plants, which enhances the plant's ability to defend itself from pathogen attack.

II. MATERIALS AND METHODS

Soil samples were collected from various sources such as region from three estuaries namely Dharmadam, Tellicherry and Mahe, belonging to the state of Kerala. Randomly located 5 X 5 m were used to attain six sub samples of the topsoil (7.5 cm depth) using an auger 8.5 cm diameter.
The samples were transported in polyethylene bags in ice pack to the laboratory. The polyethylene bags used for Collection of samples were cold-sterilized in UV-radiation box for at least 12h, while glassware was treated in the hot-air oven at 160^{o}C for 2 h. Growth media and diluents (distilled water) were autoclaved at 121^{o}C for 15 min.

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A. Soil pH was Determined According to the Procedure Described by Akpor et al. (2006).

The material must be separated on the ¼ in. (6.3 mm) sieve. Only the minus ¼ in. (6.3 mm) material is to be used for testing. Weigh and place 20 gm of soil into the glass beaker. Add 20 ml of distilled water to the soil sample. Stir to obtain soil slurry and then cover with watch glass. The sample must stand for a minimum of one hour, stirring every 10 to 15 minutes. This is to allow the pH of the soil slurry to stabilize. After one hour, the temperature of the sample should be stabilized. Measure the temperature of the sample and adjust the temperature controller of the pH meter to that of the sample temperature. Standardize the pH meter by means of the standard solutions provided. Place the electrode(s) into the soil slurry solution and gently turn beaker to make good contact between the solution and the electrode(s). Read and record the pH value to the nearest tenth of a whole number.

B. Isolation of Microorganisms from Soil

The soil sample was mixed, and a suspension of 1 g (dry weight equivalent) in 10 ml of sterile water was prepared. One ml of the soil suspension was then diluted serially (ten-fold) and used in the estimation of aerobic heterotrophic bacterial and fungal populations by standard spread-plate dilution method described by Seeley and Van Demark (1981), in triplicate. Pure isolates of representative communities were maintained on agar slant at 4^{o}C. Identification of isolates was based on cultural, microscopic, and biochemical characteristics with reference to Bergey's manual of determinative bacteriology (1989) for bacteria.

1) Isolation of Bacteria

a) Nutrient Agar: 15 to 20 ml of molten nutrient agar medium was prepared and poured into sterile Petri dishes under aseptic condition. Nutrient agar containing 0.015% (w/v) nystatin (to inhibit fungi growth) was used for bacteria isolation Nutrient agar containing 0.015% (w/v) nystatin (to inhibit fungi growth) was used for bacteria isolation and incubation was at 35^{o}C for five days. Serially diluted samples were inoculated into the plate by spread plate method. The plates were incubated at 37˚C for 24 h. Then the plates were observed and the results were recorded.

C. Isolation and Characterization of Bacterial Isolates of Interest

Depending upon the colony morphology of various isolates on Nutrient agar, they were subjected to grow on various selective media for the isolation of the bacteria of interest that is with good biocontrol activity against Fusarium Sp. We looked specifically for the presence of two bacterial isolated namely Pseudomonas and Serratia for their ability to produce Chitinase enzyme, which could inhibit the fungal growth.

D. Growth on Selective Media: Citrimide Agar for isolation of Pseudomonas (King et al,1954)

Citrimide agar of Himedia was used for the selection of Pseudomonas Sp. 45.3 g of the Cetrimide media was weighed and mixed with 10 mL of glycerol in one liter of purified distilled water. The conical flask was heated with frequent agitation and was boiled for one minute to completely dissolve the medium. Autoclaving was done at 121°C for 15 minutes to sterilize the medium. Bring the media to about 45^{0}C, and pour into the sterile petridish under sterile conditions inside the laminar air flow chamber. Allow the medium to solidify for 15 to 20 min.The soil samples were serially diluted as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and $10^{-5,}$ using sterile distilled water.0.1ml of the different dilution of samples were poured on to different plates containing media. Incubate the plates for 24 hrs at 37^{0}C in an incubator.

E. CT-Agar for Isolation of Serratia (Allen and Conger, 1969; Altemeier et al, 1969)

A defined agar medium (CT5 agar containing 0.01% yeast extract, 0.1% Caprylic n-octanoic acid and 0.025% thallium sulfate) is highly selective for all Serratia species and effectively discriminates against most non-Serratia strains likely to be in the same habitats. The selectivity of CT agar is demonstrated by the very high efficiency of colony formation on CT agar by Serratia strains.The soil samples were serially diluted as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and $10^{-5,}$ using sterile distilled water. 0.1ml of the different dilution of samples was poured on to different plates containing media. Incubate the plates for 24 hrs at 37^{0}C in an incubator.

1) Gram Staining: Individual colonies were selected and smeared over a clean glass slide. The smears were stained with Gram staining reagent, and the results were observed under light microscope.

2) Spore Staining: Selected individual colonies were smeared over a clean glass slide and stained with malachite green and saffranin, and observed under light microscope.

3) Capsular Staining: Tongue like smear was prepared and air-dried, further stained with Indian ink and observed under light microscope.

4) Motility: Motility test was performed by hanging drop method and examined under oil immersion.

F. Biochemical Characterization (Holt et al., 1994)

1) *Catalase Test:* A clean glass slide was taken; one drop of hydrogen peroxide reagent was placed at the center of the slide. A loopful of test culture was suspended in to the hydrogen peroxide reagent, it was mixed well with the help of an applicator sticks and the results were observed after 5 minutes.
2) *Oxidase Test:* A clean glass slide was taken and oxidase disc was placed at the center of the slide. A loopful of test culture was placed on it. Results were observed after 5 minutes.
3) *Indole Production Test:* Sterile tubes containing tryptophan broth were inoculated with a loopful of selected individual colonies. The tubes were incubated for 24 h at 37˚C. After the incubation period 0.5ml of Kovac's reagent was added. Then the tubes were allowed to stand for 5 minutes and examined. Results were noted.
4) *Methyl Red – Voges Proskauer Test:* Tubes containing MR-VP broth were inoculated with a loopful of selected test organisms and incubated at 37˚C for 24 to 48 hours. After incubation 5 to 10 drops of methyl red solution was added for the detection of mixed-acid production and Barrit's reagent was added to check out butylene glycol and acetoin production.
5) *Citrate – Utilization Test:* Simmon citrate agar medium was prepared and poured into clean test tubes. Selected colonies were inoculated into the slants and further incubated at 37˚C for 24 h to detect the citrate utilization. Results were recorded.
6) *Triple-Sugar Iron Agar:* Selected colonies were inoculated into the tubes containing triple-sugar iron agar by means of stab and streak inoculation to detect sugar fermentation and H_2 S production. Tubes were incubated at 37˚C for 24-48 h and the results were recorded.
7) *Carbohydrate – Fermentation Test:* Overnight isolated cultures were inoculated into carbohydrate broth containing different sugars such as glucose, arabinose, cellobiose, mannitol, mannose, raffinose, ribose, lactose, trehalose, sucrose, maltose, etc., Tubes were incubated at 37˚C for 24 h. (Kim et al., 1999).
8) *Gelatin Hydrolysis Test:* Selected colonies were inoculated into the plates containing Gelatin agar media by means of streak inoculation to detect hydrolysis. Plates were incubated at 37˚C for 24-48 h and the results were recorded.
9) *Starch Hydrolysis Test:* A loopful of test Culture was inoculated on to a Starch Agar plates and the plates were incubated at 37◦C for 24 hrs. After 24hrs Iodine Solution was poured on to the plate and zone of hydrolysis were measured and results were recorded.

G. Analyzing the Chitinase Production by Different Bacterial Strains (Harman et al, 1993)

The selected isolates from the samples were diluted to 10^1 - 10^5 fold in sterile water and were inoculated by spread plate technique on raw chitin containing minimal agar plates containing Colloidal chitin 12 g, $(NH_4)_2$ SO_4 - 2g, KH_2 PO_4 - 0.7g, Na_2 HPO_4. $7H_2O$ - 0.2g, $FeSO_4.7H_2O$ - 1 mg, $MnSO_4$ $5H_2O$ - 1 mg, Agar - 15g, distilled water - 1000ml and, pH - 7.0(Park et al., 2000). After incubation for 7 days at room temperature, clear zone forming bacteria were selected for the further study.

1) *Analysis of Chitinase Producing Activity of Bacteria:* The isolates were pre-cultured in chitin containing minimal broth for 3 days at 30°C with stirring at 150 rpm. The medium (550 ml) was inoculated with approximately 1% of pre-cultured isolates in an Erlenmeyer flask and incubated for 3 days at 30°C on a reciprocal shaker. After incubation results were observed and recorded. Organisms having more chitinase activity were selected for enzymatic assay.
2) *Measurement of Chitinase Activity of Bacteria (Harman et al., 1993):* Chitinase activity was measured by the reduction of turbidity of a suspension of colloidal chitin. A suspension containing 20 % (w/v) of moist colloidal chitin with moisture of 88% was prepared in 50 mM potassium Phosphate buffer, pH 6.7 .A mixture consisting of 0.5 ml each of the chitin suspension and the two culture solution to be tested was prepared and incubated for 24 hr. at 30^0C .The mixture was then diluted with 5 ml of water and the optical density was read at 510 nm. Activity was calculated as the percentage of reduction of turbidity relative to that of a similar suspension that contained water instead of Culture organisms. one enzyme unit was defined as the amount of enzyme required to reduce the turbidity of a chitin suspension by 5%.
3) *Purification of Chitinase enzyme (Harman et al., 1993):* The culture filtrates were dialyzed against 50 mM potassium phosphate buffer,pH6.7, overnight at 4 ^{0}C with stirring. The dialysis tubes were then placed in polyethylene glycol (35000 molecular weight) at room temperature until the volume was reduced 15 to 25 fold. Subsequently it was injected into a chromatography column (5 x 60 cm) packed with Sephacryl S-300.The column was equilibrated and eluted with 50 mM potassium phosphate buffer, pH 6.7, containing 200 mM NaCl and 0.02 % $NaNO_3$.Samples and elution buffer were pumped from the bottom of the column at a rate of 2.5 ml/min and fractions were collected every 5 min. Fractions exhibiting activity of various enzymes were pooled and analysed.

III. RESULTS

Table 1. Occurrence and abundance of aerobic heterotrophic bacteria of sampling location. Tellicherry, Dharmadam and Mahe

Bacteria	Occurrence			Abundance		
	Tellicherry	Dharmadam	Mahe	Tellicherry	Dharmadam	Mahe
Pseudomonas	+	+	+	7	8	7
Serratia	+	+	+	4	6	3

A. Evaluation Of Chitinase Producing Bacteria

Bacteria	Occurrence			Abundance		
	Tellicher ry	Dharmadam	Mahe	Tellicherry	Dharmadam	Mahe
Pseudomonas	+	+	+	6	5	3
Serratia	+	+	+	5	7	3

B. Isolation Of Chitinase Producing Bacteria

Characteristics	Pseudomonas aeruginosa	Serratia marcescens
Gram's reaction	Gram negative rods	Gram negative rods
Colony morphology	Smooth and greenish in color	Opaque and red in color
Oxygen requirement	Aerobic	Facultative anaerobic
Motility	Motile with single flagella	Motile with lateral flagella
IMViC	_	++
Carbohydrate utilization		
Maltose	Negative	Positive
Glucose	Positive	Positive
Lactose	Negative	Negative
Sucrose	Negative	Positive
Glycerol	Negative	Positive
Gelatin hydrolysis	Positive	Positive
Starch hydrolysis	Negative	Negative
Tween 80 hydrolysis	Negative	Positive
Denitirification	Positive	Positive
Catalase	Positive	Positive
Oxidase	Positive	Positive
L Lysin	Positive	Positive
L Ornithine	Positive	Positive
D Arginine	Negative	Negative
Chitin hydrolysis	Positive	Positive

C. Growth on Selective media: Citrimide Agar for isolation of Pseudomonas

Plates were examined for the presence of characteristic blue, blue-green, or yellow-green pigment. Pseudomonas typically produces both pyocyanin and fluorescein.

D. CT-Agar for isolation of Serratia

Typical Red coloured colonies were observed on the CT agar, thus indicating that Serratia was present in the soil samples.

E. Measurement of Chitinase activity of bacteria (Harman et al, 1993)

SNo	Isolate	O.D of water at 510 nm	O.D of culture at 510 nm
1	Pseudomonas Sp	1.6	1.0
2	Serratia Sp.	1.9	0.82

Activity of chitinase= O.D of water /O.D of culture after inoculation X100

Pseudomonas =1.8 / 1.0 X 100 = 180 %
one enzyme unit = the amount of enzyme required to reduce the turbidity of a chitin suspension by 5%.
200 / 5 = 36 mU/ml
Serratia =1.8 /0.84 X 100 = 214 %
214 / 5 = 42 mU/ml

IV. DISCUSSION

Tomato cultivation was affected by a number of diseases. Among them, the wilt disease caused by F. oxysporum is a serious disease in major tomato-growing areas .Talc-based formulations of plant growth promoting rhizobacteria Pseudomonas fluorescens (Pf1) and Bacillus subtilis either single or mixed along with or without chitin and neem amendments were developed and tested under greenhouse and field conditions was studied by (Bharathi et al., 2004)

Chitin is a polymer of ß – 1, 4 linked N-acetyl glucosamine and it is the main structural component of cell walls of fungi, insect exoskeletons and the shells of crustaceans (Park et al., 2000) . It is the second most abundant natural polysaccharides after cellulose on Earth. Chitin hydrolysates can be utilized as major sources of carbon and nitrogen in the production of single cell proteins (Revah Moiseer and Carrod, 1981) .

Two most important enzymes, chitinase and N-acetyl glucosaminidase are vital for total degradation of chitin. Chitinase which is highly abundant in nature hydrolyses the glycosidic linkages in chitin (Takayanagi et al., 1991) . Chitinases is having tremendous potential to be used as important bio-control agents (Zhou et al.,1999) particularly as an antifungal agent through chitin degradation activity (Mathivanan et al., 1998).

Most abundant of chitinase producing bacteria are present in marine surroundings like Aeromonas sp., Clostridium sp., Vibrio sp., Streptomyces sp. and some other microorganisms.

Chitinase purification and characterization, molecular cloning and expression, family and structure, regulation, and function and application also been studied (Duo-Chuan L,2006).Hence an attempt has been made to segregate and categorization the chitinase producing bacteria from estuaries of Arabian sea.

Based on the morphological and physiological characteristics and results of MIS of the isolated bacteria were identified and confirmed as Serratia Sp.and Pseudomonas Sp.

This study shows only the segregation and categorization of chitinase producing bacteria from estuaries of Arabian sea.This is one of the reports of the isolation of chitin degrading bacteria such as Serratia and Pseudomonas from estuaries of Arabian sea. When the crude chitin containing minimal broth inoculated with Serratia Sp. and Pseudomonas Sp. showed high chitinase activity with no culture growth due to poor nutrition, where as the medium containing other nutrient sources like glucose, glycerol and N-acetylglucosamine showed rapid growth of Serratia Sp., but the other bacteria, Pseudomonas showed only slow growth in the medium supplemented with glucose and N-acetylglucosamine due to lack of glycerol utilizing activity.

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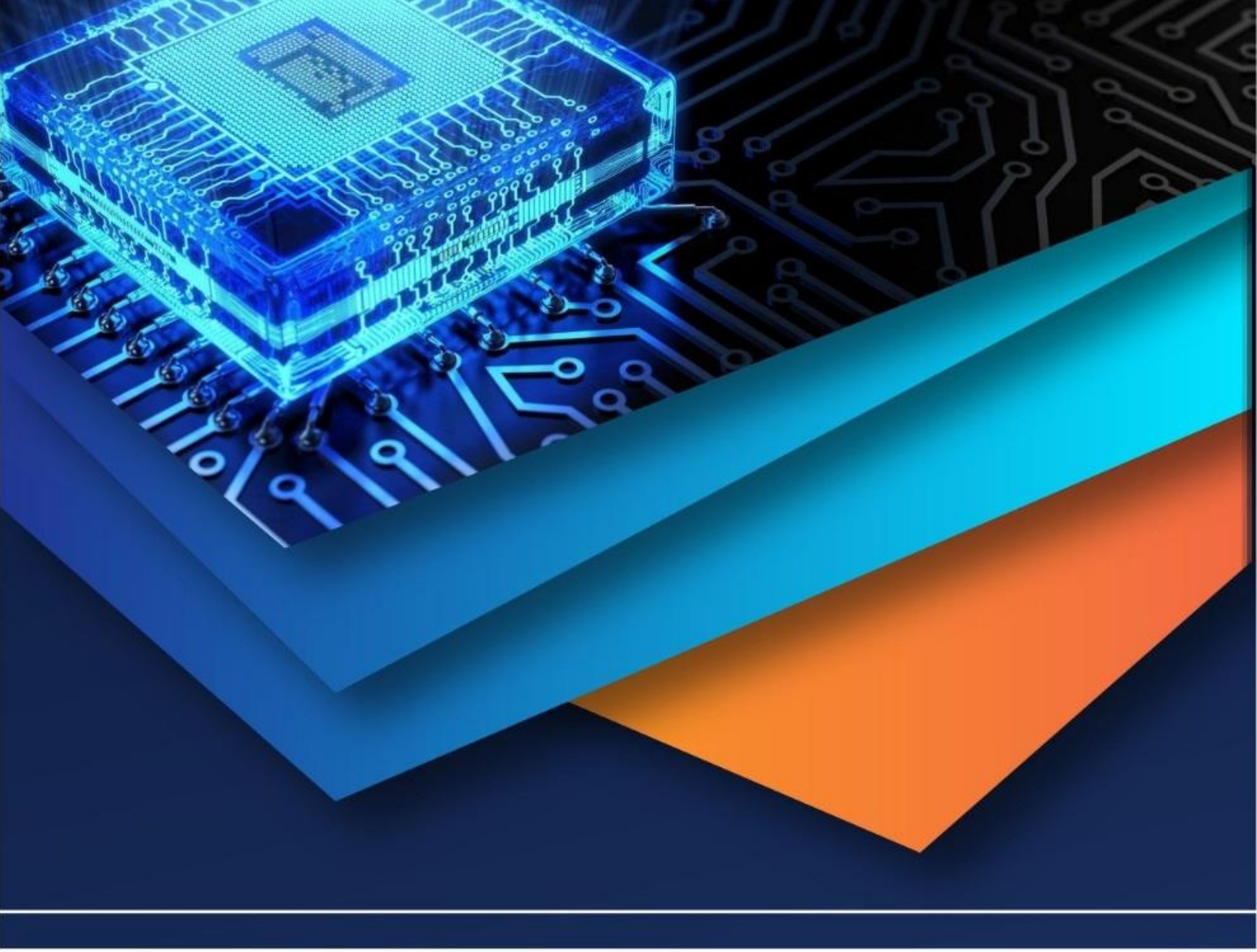

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