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Research Article

IN VITRO ANTAGONISM AND EVALUATION OF CHITINASE ACTIVITY OF BACTERIA– *BACILLUS CIRCULANS* AGAINST PATHOGENIC FUNGI IN *VIGNA UNGUICULATA*

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ABSTRACT: Scientists of agriculture and plant pathology are on the lookout for potential biological control agents to control the plant pathogenic organisms in order to avoid soil contamination. Rhizospheric bacteria are excellent agents to control soil-borne plant pathogens. In this study an attempt has been made to evaluate the antagonistic activity of a bacterial strain *Bacillus circulans* against *Curvularia lunata*, *Alternaria alternata* and *Cladosporium sp.,* which are important seed and soil borne pathogens distributed throughout the world. These infections cause grain mold and leaf spot diseases, resulting in significant economic loss to the crops. A soil bacterium *Bacillus circulans* isolated from the rhizospheric soil of *Vigna unguiculata*, showed high antagonistic activity against *Curvularia lunata*, *Alternaria alternata* and *Cladosporium sp*. on dual plate assay. *Bacillus circulans* showed a distinct inhibition zone in the dual plate assay and also produced a clear inhibition zone in chitin amended agar medium containing 0.5% colloidal chitin, indicating that it secretes chitinase.

Key words: Antagonistic activity, fungal pathogen, *Bacillus circulans*, chitinase

INTRODUCTION

Plant pathogenic fungi cause many dreadful diseases in plants throughout the world. Hence it is crucial to eliminate or to control the plant pathogens so as to increase the quality and yield of the crop. Synthetic chemical based fungicides were extensively used to control fungal diseases however they have deleterious effects on the surrounding environment. The use of synthetic fungicides and other methods were not found to be successful in eradicating these harmful pathogens. Recently, bacterial species have been used in controlling fungal diseases. These micro organisms are capable of lysing chitin, a major component of the fungal cell wall, thus playing an important role in biological control of fungal pathogens. Fungi like *Trichoderma*, and bacteria like *Bacillus*, *Serratia, and Alteromonas* were reported to have chitinolytic activity (Ashwini and Srividhya, 2014). Non-pathogenic soil bacillus species offers several advantages over other organisms as they form endospores and hence can tolerate extreme temperature, pH and osmotic conditions. *Bacillus* species were found to increase plant growth and cause lysis of fungal mycelia by colonizing the root surface.

MATERIALS AND METHODS

Sample Collection: The infected plants (*Vigna unguiculata*) and its own rhizosphere were collected from an agricultural farm in Padalam near Vedanthangal and pathogenic fungi were isolated and grown on potato dextrose agar.

Preliminary In vitro Antagonism test: Ten isolates of Bacteria were obtained from *Vigna unguiculata* rhizosphere and, were evaluated in vitro as antagonists against isolates of *Alternaria alternata*, *Curvularia lunata* and *Cladosporium* sp. Dual cultures were carried out by using one-week-old cultures of *Alternaria alternata*, and *Curvularia* sp. on PDA. A 5-mm-diameter disc of the antagonist was positioned opposite a 5-mm-diameter disc of the pathogen on the inoculated agar medium. An approximate distance of 5 cm was maintained between discs. Cultures were grown at 29 ± 2°C, and observed after four days. A sterile disc of agar was placed in the control treatment plate instead of the bacterial isolates. Replicates of three were used for each treatment. At the end of the incubation period, radial growth was measured. The efficiency of Bacteria in suppressing radial growth was calculated as follows: (C _ T)/C · 100 where C is radial growth measurement of the pathogen in the control and T is radial growth of the pathogen in the presence of the test Bacterium.

Identification of bacterium: The identification of bacterial isolate A002 was carried out by the methods suggested in the Bergey's Manual of Systematic Bacteriology (Williams *et al.,* 1989).

Screening for enzyme production: Colloidal chitin was prepared from the chitin flakes by the method of Mathivanan (1995).

Qualitative assay of Chitinase: The selected bacterial culture was isolated and streaked on a chitin agar plate (colloidal chitin 0.5%, agar 1.5%). The isolate was inoculated in Nutrient broth (NB) amended with 1% of colloidal chitin. One ml of each test bacterial inoculums was inoculated to 100 ml of medium and incubated at 100 rpm in a rotary shaker at room temperature. After two days of incubation, the culture was harvested, centrifuged at 10000 rpm for 15 min at 4^{o}C and the supernatant was collected. Colloidal chitin (0.5%) agar plates were prepared and the antagonistic bacteria A002 was streaked on the plate filtrate and incubated at 37^{o}C. After 24h, the development of clear zone around the streaked colony was observed. Protein content in the supernatant was quantified by using the method of Bradford (1976).

Chitinase assay: Chitinase activity was assayed by the colorimetric method of Reissig (1955). Chitinase activity was determined using N-acetylglucosamine as the standard. One unit of chitinase activity was defined as the amount of enzyme, which produces 1 µ mole of N-acetylglucosamine in 1 ml of reaction mixture under standard assay condition

Optimization of culture conditions: In order to obtain maximum chitinase production, 1 % colloidal chitin was amended to three types of media, namely NB, Luria Bertani broth (LB) and yeast nitrogen base broth (YNB). A twenty-four hour old inoculum of A002 with 0.5 OD was inoculated in 100 ml of each medium and incubated at 150 rpm in a rotary shaker at room temperature. The cultures were harvested and centrifuged at 10000 rpm for 15 min after two days of incubation. The supernatant was then used to assay chitinase.

A002 was grown in NB broth at different concentrations (0.1 - 0.5%) of colloidal chitin to determine the optimum concentration of substrate for chitinase production. Similarly, it was grown at different pH (5 -8) in NB amended with 0.5% of colloidal chitin and at different temperatures (20 – 40^{o}C) to determine the optimum pH and temperature for chitinase production. After two days of growth, the cultures were harvested, centrifuged and the supernatant was used to quantify chitinase.

A002 was grown in NB broth with optimized growth conditions (0.5% colloidal chitin, pH 5.5 and temperature 35^{o}C) for 3 days. At every six hour interval, the production of chitinase in the culture filtrate was assayed.

Production of Enzyme Extract: Bacterial cells were removed by centrifugation at 10,000 rpm for 25 min and the supernatant was used as enzyme source.

Determination of Chitinase activity by electrophoresis: The partially purified enzyme product was used to demonstrate chitinolytic activity on polyacrylamide gel. The sample buffer without β-mercaptoethanol and containing 50µg of protein was loaded onto a 10% Sodium dodecyl sulphate poly acrylamide gel and this gel was subjected to electrophoresis at a constant current (100 volts). After the gel was run, it was incubated for 5 hours in 100mM sodium acetate buffer at pH 5.2 with 1% triton-x100.After which, a 7% acrylamide gel overlay containing 0.2% colloidal chitin was placed on either side of the gel. This was allowed to incubate overnight at 37^{o}C. The following day, the gel was stained with silver nitrate.

RESULTS AND DISCUSSION

Isolation and Screening:

In this study, 10 bacterial isolates and 15 fungal strains were isolated from the rhizosphere and infected regions of *Vigna unguiculata* respectively. The fungi were identified based on their morphology and 3 strains which were found to be the major pathogens of *Vigna unguiculata* are listed in the table below

Table 1: Isolated Fungal Pathogens

S.No	Pathogens identified
1.	*Alternaria alternate*
2.	*Curvularia lunata*
3.	*Cladosporium* sp.

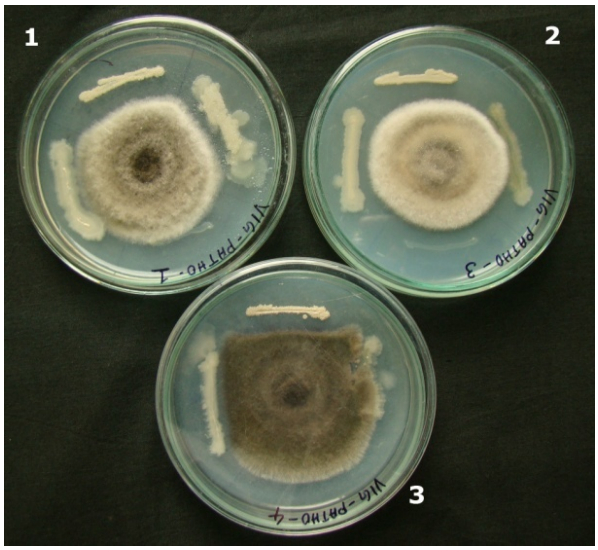

Fig 1: Plate 1 Antagonistic effect of test bacterium on *Alternaria alternata,* Plate 2 Antagonistic effect of test bacterium on *Cladosporium* sp., Plate 3 Antagonistic effect of test bacterium on *Curvularia lunata*

Of the 10 strains of fungi, 6 strains were found to possess antagonistic properties against the mentioned pathogenic strains of fungi. The strain with the highest inhibition ratio was selected. Inhibition Ratio = (C _ T)/C · 100 Where is radial growth measurement of the pathogen in the control and T is radial growth of the pathogen in the presence of Bacteria.

Table 2: Evaluation of antagonism of isolated bacteria against pathogenic fungi

S.No	Bacterial Isolate	*Alternaria alternata*		*Curvularia lunata*		*Cladosporium* sp.	
		Growth area mm	Inhibition Ratio	Growth area mm	Inhibition Ratio	Growth area mm	Inhibition Ratio
1.	A001	4.0	57.9%	3.6	60.9%	3.7	61.5%
2.	**A002**	**1.6**	**83.2%**	**1.0**	**89.1%**	**1.2**	**87.5%**
3.	A003	9.5	0.0%	9.2	0.0%	9.6	0.0%
4.	A004	5.2	45.3%	5.0	45.7%	4.9	48.9%
5.	A005	3.8	60.0%	3.3	64.13%	3.5	63.5%
6.	A006	3.7	61.0%	3.2	65.2%	3.4	64.5%
7.	A007	9.4	1.0%	9.2	0.0%	9.6	0.0%
8.	A008	3.0	68.4%	2.6	71.7%	2.8	70.8%
9.	A009	9.3	2.1%	9.2	0.0%	9.6	0.0%
10.	A010	9.5	0.0%	9.2	0.0%	9.6	0.0%
Control		9.5	0.0%	9.2	0.0%	9.6	0.0%

General characteristics: Semi-transparent colonies of varying size. As the colonies grow they become less transparent. The bacterium was found to be Gram- negative, motile, large spore-forming rods and catalase –positive. Based on all the biochemical tests and morphological characters, the bacteria were identified as *Bacillus circulans.*

Qualitative assay for chitinase: a clear hydrolytic zone was visible around the bacterial culture in the chitinase plate. This zone indicated that the bacterium was capable of utilizing the substrate chitin. After 48 hrs of incubation, the qualitative screening resulted in clear hydrolytic regions 20mm in diameter, around the culture colony. Thus the test bacilli screened and selected for the production chitinase was capable of degrading the substrate chitin, which is a major structural component of fungi cell walls.

Optimization of media: Nutrient broth amended with1% colloidal chitin produced the highest amount of chitinase, in comparison with Luria Bertani and Yeast Nitrogen Base.

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Table 3: Biochemical nature of the test bacterium

S.No	Test	Result
1	Gram staining	-ve
2	Motility	+ve
3	Citrate utilization	-ve
4	Urease	-ve
5	Catalase	+ve
6	Glucose	+ve
7	Sucrose	+ve
8	Starch	+ve
9	Chitin	+ve
10	Gelatin	+ve
11	Indole	-ve
12	Nitrate	+ve
13	Endospore	+ve

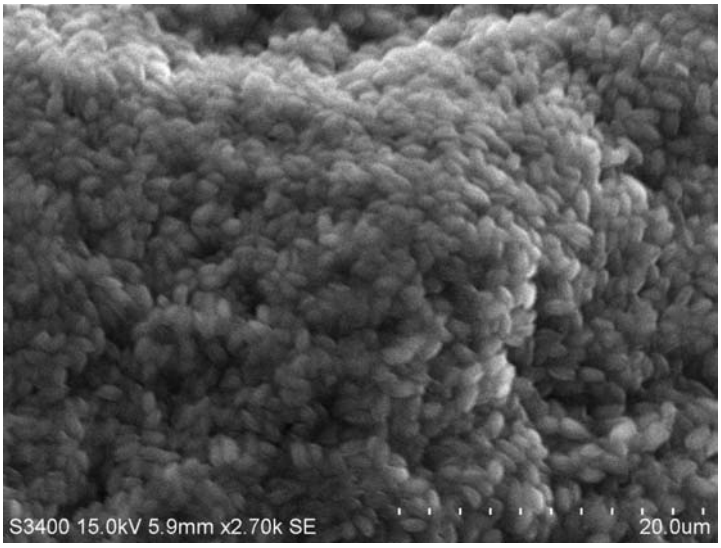


Fig 2: Scanning electron microscope image of *Bacillus Circulans* colony.

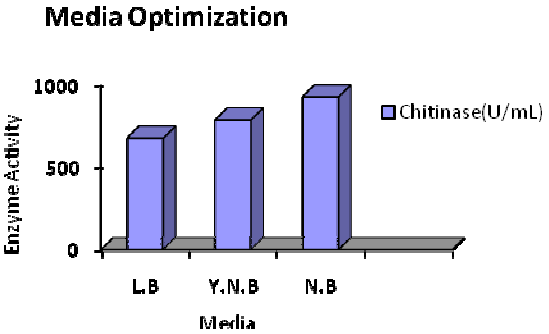


Fig 3: Media optimization

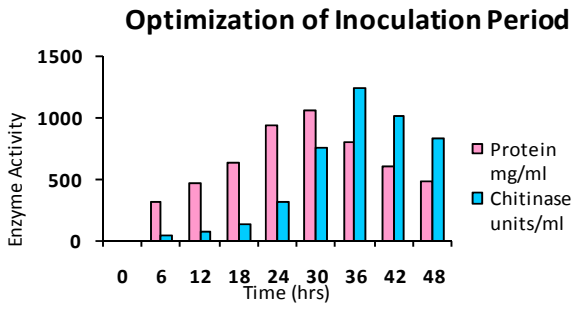


Fig 4: Incubation period optimization

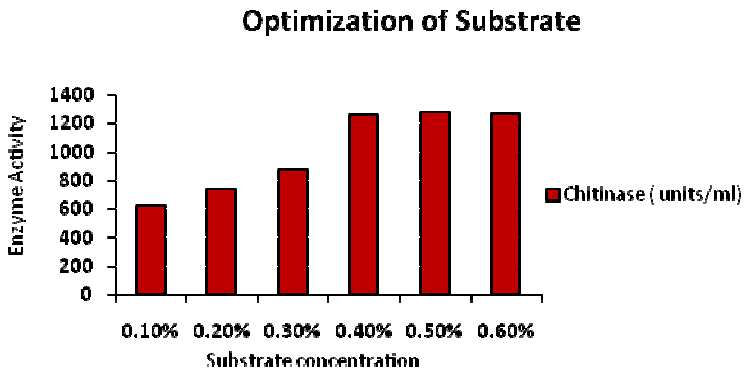
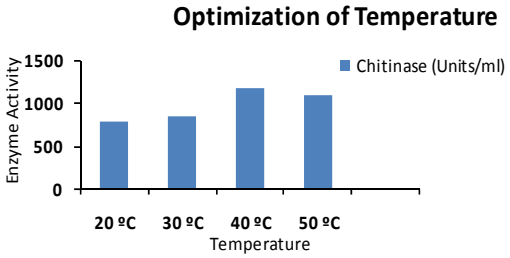


Fig 5: substrate concentration optimization



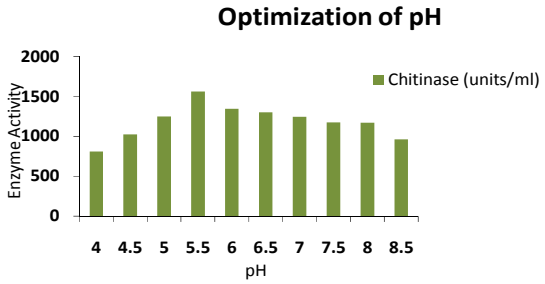


Fig 6: Temperature optimization **Fig 7: pH optimization**

From the given table, we infer that optimum enzyme is produced at the pH of 5.5, with the substrate of 0.5% colloidal chitin. The preferred medium is nutrient agar amended with 0.5% colloidal chitin at a temperature of 40^{o}C for 42 hrs.

Electrophoresis: The purified chitinase was subjected to zymogram analysis and hydrolyzed bands indicated the presence of chitinase activity.

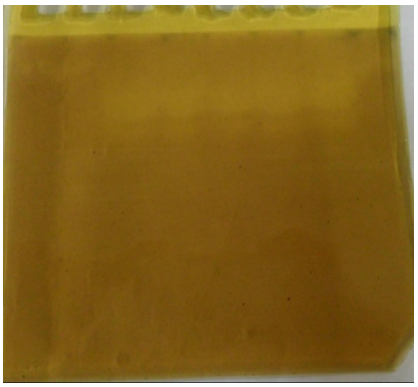


Fig 8: Activity Page demonstrating the activity of enzyme chitinase

DISCUSSION

The disease control mechanisms currently used to prevent and control fungal infections mostly involve repeated spraying of chemicals combined with the labor-intensive practices of canopy management. The use of chemicals is environmentally unfriendly and very expensive. Building up of resistance in the pathogen population is always a concern.

The application of chemicals is becoming increasingly undesirable due to consumer insistence on healthier and more naturally produced foods. Moreover, the long-term adverse effects of agricultural chemicals on the environment are well-known and it is generally accepted now that the agricultural community should scale down their dependence on these products. In the last decade, there has been increasing interest in transgenic approach to control diseases. On an average, two or three antibiotics obtained from bacterial sources enter the market every year.

Similarly, bio-control agents derived from bacteria are now being developed to avoid the usage of chemical fertilizers. (Nazim, *et.al*, 2008). The production of biocontrol compounds depend on a number of parameters such as physiological characters, taxonomical position, geographic location, soil composition, etc. Thus a number of bacteria have to be screened in order to maximize the chance of finding an effective biocontrol agent with broad spectrum antifungal activity.

In this study, we have attempted to isolate a bacteria capable of controlling the destruction of *Vigna ungulata* crops Isolation of 10 bacterial isolates and 15 fungal isolates from the rhizosphere and infected plants of Vigna unguiculata were obtained. Only 3 fungal isolates proved to be pathogenic and were identified as Curvularia lunata, Alternaria alternata and Cladosporium sp. 6 of the bacterial isolates possess antagonistic activity. The isolate A002, showing the highest inhibition ratio 86.6% was selected for further investigation. Isolate A002 was identified as Bacillus circulans based on its morphology and biochemical nature. Many researchers have reported that, the genera *Bacillus*, *Pseudomonas*, *Agrobacterium* and *Streptomyces* are the main bacterial genera with the capacity to produce antifungal bioactive metabolites (Ongena M. *et al.,* 2007; Raaijmakers JM *et.al.,* 2002) .The most important genus with antagonistic activity being Bacillus ,comprises around 80.0% of the inhibitory isolates in the study of Ranjbariyan (2011) . *B. subtilis* is the most important species within *Bacillus* species, and to some extent other species such as *B. amyloliquefaciens* and *B. valismortis* have been reported to produce a wide range of structurally related antimicrobial compounds these strains of bacteria are usually isolated from the soil , in their natural habitat (Stein T. 2005; Arrebola E, *et al.,* 2010; Akhavan 2007; Zhao Z *et.al.,* 2010). Mitchell and Alexander (1962) have demonstrated that the addition of chitin to soil has reduced pathogenic fungal populations and parasitic nematodes by increasing populations of chitinolytic bacteria, especially actinomycetes and fungi. Previous Studies have reported that when a plant is invaded by a pathogen, it produces certain proteins such as chitinases and b-1, 3-glucanases, and the putative role of both chitinases and b-1, 3-glucanases in response to the attack and in the case of disease resistance it is related to their capability to degrade fungal cell walls, mainly composed of chitin and b-1, 3-glucan (Joosten *et al.,* 1995). Previous studies have shown that chitin can increase the population of several biocontrol bacteria, which was closely associated with the high level of decay control (Kokalis-Burelle *et al.,* 1992; Bell *et al.,* 1998).The chitinase activity is usually induced in the presence of the chitin, which may have diverse biological roles including the antifungal activity (Dahiya *et al.,* 2006; Gohel *et al.,* 2006; Li, 2006).Increases in such microbes are correlated with reductions in pathogenic fungi and nematodes (Mankau and Das, 1969).Thus the bacteria was tested for the production of enzyme chitin. Qualitative and quantitative assays proved that chitin was indeed produced by the bacteria and this could be a potential reason for its antagonistic activity on the pathogenic fungi. *Bacillus circulans* produced extracellular Chitinase. Maximum chitinase was produced in a 42 hr old culture at 40 C in the preferred media was nutrient media amended with 0.5% colloidal chitin at pH 5.5. The chitinase SDS PAGE activity gel showed 2 distinct hydrolyzed regions, thus indicating that more than one type of chitinase is produced by *Bacillus circulans* extracellular.

CONCLUSION

Further studies have to be carried out to identify, purify and isolate other bacterial metabolites which may be used as effective biocontrol agents. Attempts are on to purify and characterize enzymes secreted by the bacterium with special attention to chitinase from the bacterial supernatant.

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