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Streptomyces sp. 9p as effective biocontrol against chilli soilborne fungal phytopathogens

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ABSTRACT

The study identified a potent Actinomycete isolate 9p with broad spectrum antifungal property against four phytopathogens tested- Alternaria brassiceae OCA3; Collectotrichum gleosporioides OGC1; Rhizoctonia solani MTCC 4633 and Phytophthora capsici. The isolate was identified as Streptomyces sp. based on colony morphology, microscopy and 16srDNA. The isolate produced 2 most important hydrolytic enzymes- chitinase and β-1, 3 glucanase along with cellulase, lipase and protease. The strain 9p produced relatively high levels of chitinase (696 U/mL) at day 4 of the incubation period. Meanwhile, the highest level of β -1,3-glucanase (392 U/mL) was also found at day 4 of the incubation period and subsequently decreased slightly during the stationary phase. Among the various carbon sources tested in the ISP media, all carbon amendments supported induction of both chitinase and glucanase. Even the autoclaved mycelia of Colletotrichum gleosporoides (OGC1) and Alternaria brassicae (OCA3) supported appreciable levels of both the enzymes. Concurrent production of protease, lipases, siderophore, IAA production coupled with anti-fungal activity suggests the plant growth promotion and broad spectrum biocontrol potential of this isolate. The strain 9p exhibited mixed path antagonism type of mechanisms of biocontrol through the production of mycolytic enzymes. Isolate showed 87.5% germination index when coated onto chilli seeds. This coupled with antifungal properties, suggests both the PGPR and biocontrol aspect of the actinomycete Streptomyces sp. 9p.

Key words: *Streptomcyces* sp, antagonism, PGPR, mycolytic enzymes, seed bacterization.

INTRODUCTION

Microbial antagonists are widely used for the biocontrol of fungal plant diseases. Many species of *Actinomycetes*, particularly those belonging to the genus *Streptomyces*, are well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi [1]. The antagonistic activity of *Streptomyces* to fungal pathogens is usually related to the production of antifungal compounds [2] and extracellular hydrolytic enzymes [3]. Chitinase and β-1,3-glucanase are considered to be important hydrolytic enzymes in the lysis of fungal cell walls, as for example, cell walls of *Fusarium oxysporum*, *Sclerotinia minor*, and *S. rolfsii* [1,4]. Potential uses of naturally occurring bacteria,

Actinomycetes and fungi replacement or supplements for chemical pesticides have been addressed in many studies [5].

Actinomycetes are Gram-positive bacteria with a high guanine plus cytosine content in their DNA (> 55 mol %). The group encompasses genera covering a wide range of morphologies extending from the coccus (*Micrococcus*) and rod-coccus cycle bacteria (e.g. *Arthrobacter*), through fragmenting hyphal forms (e.g. *Nocardia*), to genera with a permanent and highly differentiated branched mycelium (*Micromonospora*, *Streptomyces* and others) [6].

Despite the well-documented history of *Streptomyces* in biocontrol and preliminary evidence of their capacity to enhance plant growth [7], *Streptomyces* species have been poorly investigated specifically for their potential as PGPR. This is surprising as *Streptomycetes*, generally accounting for an abundant percentage of the soil microflora, is particularly effective colonizers of plant root systems and is able to endure unfavorable growth conditions by forming spores [8]. Merriman *et al.,* (1974) [9] reported the use of a *Streptomyces griseus* (Krainsky), Waksman and Henrici isolate as a seed treatment of barley, oat, wheat and carrot, in order to increase their growth. The isolate was originally selected for the biological control of *Rhizoctonia solani*. Though the *S. griseus* isolate did increase the average grain yield, dry foliage weight, tiller number, and advanced head emergence for wheat and oat over controls, the differences were not statistically significant. Nearly 20 yr later, though studies by El-Abyad *et al.* [1993] and Merriman *et al.* [1974] reported plant growth enhancement as a function of inoculation with *Streptomyces*, they did not investigate the possible mechanisms of *Streptomycete*- mediated growth promotion [9,10].

The Solanaceae represent the third most economically important plant taxon, and the most valuable in terms of vegetable crops with agricultural utility [11], representing more than 3000 species, including the tuber-bearing potato, a number of fruit-bearing vegetables (tomato, eggplant, peppers), ornamental plants (petunias, Nicotiana), plants with edible leaves and medicinal plants.

The present study demonstrates the efficiency of Actinomycetes isolated from Solanaceae rhizosphere to produce lytic enzymes viz. Chitinase, β-1, 3 and β-1,4 glucanase, lipase and protease in addition to their possible role in the destruction of broad spectrum soil borne fungal phytopathogens- *Alternaria alternata* OTA36; *Alternaria brassicola* OCA1; *Alternaria brassiceae* OCA3; *Collectotrichum gleosporoides* OGC1; *Rhizoctonia solani* and *Phytophthora capsici*. The PGPR traits of the organism were determined in terms of IAA, siderophore, PO_4 solubilization etc. and germination index of chilli. The antifungal potential of extracellular mycolytic enzymes produced by soil-borne Actinomycete could be exploited for its future use as a biofungicide.

MATERIALS AND METHODS

Isolation of rhizospheric Actinomycete

Actinomycetes were isolated from Solanaceae rhizospheric soils like Brinjal, Capsicum and Chilli grown in Bangalore and Assam by soil dilution method. The different isolates obtained on ISP-4 medium containing were screened for chitinase production on chitin agar plates according to Chernin *et al*., (1995) [12]. The 9p culture was maintained in ISP medium.

Preparation of colloidal chitin

Chitin from crab shell was used for the preparation of colloidal chitin. Colloidal chitin was prepared from purified chitin according to the method of Roberts and Selitrennikoff (1988) [13].

Detection of Hydrolytic Enzymes

Estimation of Extracellular Chitinase Activity

For qualitative estimation, Chitin agar plates [chitin from crab shell (Sigma)] with ISP medium were prepared. Actinomycetes cultures were spot inoculated and incubated at 28°C. After 7 days of incubation, zone of inhibition was observed around the actinomycetes colony [14].

Chitinase Enzyme assay
Chitinase activity was measured with colloidal chitin as a substrate. The culture broth was centrifuged and enzyme solution 1 ml was added to 1.0 ml of substrate solution, which was made by suspending 1% of colloidal chitin in Phosphate buffer (pH 7.0). The mixture was incubated at 37°C for 45 minutes and the amount of reducing sugar produced in the supernatant was determined by DNS method [15]. One unit of chitinase activity was defined as the amount of enzyme that produced 1 µmol of reducing sugar per min **[**16].

Estimation of extracellular Glucanase Activity
Plates with ISP containing (1%, w/v) Carboxy Methyl Cellulose (CMC) were prepared. 9p was spot inoculated in the centre of the plate. After an appropriate incubation period at 28°C, the agar medium was flooded with an aqueous solution of Congo red for 15 min. The Congo red solution was then poured off, and plates containing CMC were visualized for zones of hydrolysis [17] detecting β-1,4 glucanase. Azo-glucan containing plates by Chen *et al*.,[1997] was used to detect β-1,3 glucanase. ISP medium with (1%, v/v) azoglucan was prepared and spot inoculated with the isolate. Deposition of blue azodye surrounding the colony indicated enzyme production [18].

Glucanase enzyme assay
The specific activity of ß-1, 3-glucanase was determined by measuring the amount of reducing sugars liberated using dinitrosalicylic acid solution (DNS) (15). The culture broth was centrifuged and enzyme solution 1 ml was added to 1.0 ml of substrate solution which contained 1 ml of Yeast Cell Wall Extract (YCW, 1%, v/v). The mixture was incubated in a water bath at 40°C for 30 min and the reaction was terminated by adding 1ml of DNS solution and incubated in boiling water bath for 10 – 15 min till the development of the colour of the end product. Reducing sugar concentration was determined by optical density at 540 nm [19].

Detection of Protease
Protease activity indicated by casein degradation was determined from clearing zones in skim milk agar (50 ml sterilized skimmed milk mixed at 55 °C with 2.5% agar) after 5 days of incubation at 28 °C [20].

Detection of Lipase
The method involves measurement of fluorescence caused by the fatty acid released due to the action of lipase on olive oil. A quantitative fluorescence lipase assay is based on the interaction of Rhodamine B with fatty acid released during the enzyme hydrolysis of olive oil [21].

In vitro screening of Actinomycetes isolates for PGPR activities.
IAA production
IAA production was detected by the modified method as described by Brick *et al*., (1991) [22]. The cultures were grown in ISP broth supplemented with Tryptophan (5mg/ml) and incubated for 4 days. On 4th day Kovac's reagent were added and the formation of cherry red ring indicates the production of IAA; or Salkowski reagent (50ml 35% of perchloric acid, 1ml 0.5M $FeCl_3$ solution was added and development of pink colour indicates the IAA production.

HCN Production
All the isolates were screened for the production of hydrogen cyanide by adapting the method of Lock (1948) [23]. ISP broth was amended with 4.4g glycine/l and actinomycete was streaked on modified agar plate. A Whattman filter paper No.1 soaked in 2% sodium carbonate in 0.5% Picric acid solution placed in the top of the plate. Plates were sealed with paraffin and incubated at 28 ±2ºC for 4 days. Development of orange red colour indicated HCN production.

Detection of the Phosphate Solubilizing Activity
Phosphate solubilizing activity was assayed on yeast extract dextrose-$CaHPO_4$ agar plates by measuring the clear zone surrounding the developed Actinomycete colony, after 7 days of incubation at 30°C [24].

Siderophore Production
Actinomycetes isolates were assayed for siderophore production on the chrome azurol S agar medium described by Schwyn and Neilands (1987) [25]. Chrome azurol S agar plates were prepared and divided in two equal sectors and

spot inoculated with test organism and incubated at 28±2ºC for 48-72h. Development of yellow-orange halo around the growth was considered as positive for siderophore production.

Phytopathogens

The following six phytopathogens obtained as a kind gift from IIHR, Hessarghatta, Bangalore, were used in the study-:- *Alternaria* brassisicola (OCA1), *Alternaria brassiceae* (OCA3) *Alternaria alternate* (OTA36), *Colletotrichum gloeosporioides* (OGC1) *Phytophthora capsici* (98-01) and *Rhizoctonia solani* (MTCC 4633) was obtained from MTCC, IMTECH Chandigarh.

Screening of actinomycetes for antifungal activity

The antifungal activity of actinomycetes was tested by agar plug method. The isolated strains were seeded over the entire surface of ISP 2 medium Petri dishes. As soon as the growth of the organism was seen, agar discs were cut out by the cork borer (6 mm diameter) and transferred to the surface of Potato Dextrose Agar plates seeded with the test organisms i.e. *Colletotrichum gleosporoides* (OGC1), *Alternaria brassicae (OCA3), Rhizoctonia solani* and *Phytophthora capsici* . Petri dishes were then kept in incubator at 27°C to allow the growth of test organisms. The plates were incubated for 5-6 days at 28±2ºC. The antifungal activity was evaluated by measuring the growth of inhibition zone against test fungi. Antifungal activity was indicative as mycelia growth of fungal isolates was prohibited in the direction of active 9p isolate [26].

The percentage of inhibition of pathogen was calculated using the formula,

Percentage inhibition=[(Control-Test)/Control] X 100

Induction of lytic enzymes of isolate 9p

9p was separately grown on an ISP medium. The ISP medium was supplemented with dead fungal mycelium (*Colletotrichum gleosporoides* and *Alternaria brassicae* pre-grown in PDB for 5 days, filtered and mycelia collected) as inducers for enzymes production at a concentration of 1g % (w/v) and dispensed flasks (250 ml) each flask contained 50 ml of medium. The flasks were autoclaved and each flask was inoculated with 1.0 ml of a pre cultured 9p. The cultures were incubated shaker incubator, at 28±2°C. Two flasks from each culture were analyzed daily for 5 days [27].

Seed bacterization

Germination efficiency and Antagonism against fungal plant pathogen was checked on Chilli (*Araka shweta*) seed *in vitro*. The water agar plates were seeded with the following: Set 1- Control-plain seed were coated with Carboxy Methyl Cellulose (CMC); Set 2-Seed coated with CMC and *Colletotrichum* spores; Set 3-Seed coated with CMC and isolate (9p); Set 4-seed coated with both *Colletotrichum* and isolate (9p); Chilli seeds were surface sterilized successively with sterile distilled water and 0.1% $HgCl_2$. To remove the residual $HgCl_2$, seeds were washed with sterile distil water .The isolate was inoculated into ISP medium broth and incubated for 4 days at 28°C. *Colletotrichum* was inoculated into PDA plates and incubated at 28 °C for 3-4 days .Upon growth of the culture, for Set 2 the *Colletotrichum* spore suspension was coated. For set 3 the broth with the isolate (9p) was coated. Similarly for set 4 the CMC coated seeds were coated with *Colletotrichum* and isolate (9p). The above 3 sets of treated seeds were seeded onto 1% water agar plates. Plain CMC coated seeds on water agar were used as a control. The 4 sets were monitored regularly for generation and growth. After 1 week, the seeds were observed for antagonism against *Colletotrichum* coated seeds by the isolate [28].

RESULTS AND DISCUSSION

Isolation and screening of chitinolytic bacteria

Screening of chitinolytic actinomycetes isolates was carried out by spread inoculum of each colony on plates containing a minimal salt medium with colloidal chitin as a sole carbon and energy source. The chitin degrading organism formed colonies of 1-2 mm in diameter, surrounded by clear zones indicating chitinase activity.

12 Actinomycetes were isolated from Rhizosphere of Solanaceae family namely Brinjal, Capsicum, Chilli and screened for the production of chitinase enzyme. Total of 5 isolates-8p, 9p, 1t4, 11p and 13a were the most potent

chitinolytic actinomycetes species. All isolates showed zone size > 10 mm and were chosen for further studies (Table 1; Plate 1).

The present investigation evaluated the potential of actinomycetes isolated from different rhizospheric soils to control a broad spectrum of fungal phytopathogens of Solanaceae crops. This study dealt with chitinase-producing actinomycetes as a promising mechanism that could be utilized as biological control agents, because chitin is a major constituent of most of the fungal phytopathogens [29]. Therefore, production of this enzyme was used as the main criterion for selection of potential biocontrol agents against this phytopathogen [1,29].

Table 1: Zone of clearance on chitin agar plates

Isolate	Zone of clearance on day 6
8p	14 mm
9p	**15 mm**
1t4	11 mm
11p	14.5mm
13a	6 mm

Plate 1: Chitin clearance zone shown by isolate 9p on chitin agar plate on day 5

Detection of hydrolytic enzymes

Detection of chitin-degrading bacteria from natural sources such as rhizosphere soil is useful in the isolation of bacteria that produce antifungal or other novel compounds. A high correlation between chitinolysis and production of bioactive compounds has been reported [30]. Microorganisms, which secrete a complex of mycolytic enzymes, are considered to be possible biological control agents of plant diseases [5]. When tested for the presence of other hydrolytic enzymes like cellulose (β-1, 4; β-1, 3), protease and lipase activity, 9p tested positive for all and 8p, 1t4, 11p tested positive for cellulose (β-1, 4; β-1, 3) and protease (Table 2; Plate 2). Chitinolytic actinomycetes previously used for *in vitro* studies have included *Streptomyces viridicans* [31], *S. halstedii* and *S. Coelicolor* [32]. Singh *et al*., (1999) used a chitinolytic *Streptomyces* sp. for the suppression of cucumber wilt caused by *Fusarium oxysporum* [33].

Detection of PGPR traits

The isolates were tested for varied levels of PGPR traits – Siderophores, HCN, phosphate solubilization and IAA. The isolates showed varied degree of PGPR traits (Table 2; Plate 3). 9p isolate detected positive for IAA and siderophore production but was negative for PO_4 solubilization and HCN production. Additionally, 9p showed β-heamolysis property which indicates the biosurfactant property.

Siderophores are usually produced by various soil microbes including actinomycetes to bind Fe^{3+} from the environment and make it available for its own growth; plants also utilize these as an iron source [34]. Actinomycetes (*Streptomyces* spp.) isolated from rhizosphere soil have been reported to produce siderophores and inhibit the growth of phytopathogens [35]. Actinomycetes found in the rhizosphere need to compete with other rhizosphere plant pathogens for iron, hence, competition for iron is also a possible mechanism to control the phytopathogens. HCN production is also reported to play a role in disease suppression [36].

IAA producing microorganisms are known to promote root elongation and plant growth [37]. By producing the plant hormones, microorganisms stimulate plant growth in order to increase the production of plant metabolites which can be beneficial for their growth. In the present investigation, two out of six antagonistic actinomycetes produced IAA suggesting that these isolates could be used for plant growth promotion.

S. purpureus and *A. ramosus* have the abilities for the production of both siderophores and indole acetic acid (IAA) as estimated by Hassanin *et al*., 2007 [38]. Narayana *et al*., 2009 screened *Streptomyces albidoflavus* for plant growth promoters like IAA [39].

***In vitro* antagonism against Phytopathogens**

Dual plate assay against few selected soil borne phytopathogens revealed anti-fungal activity by isolate 9p against *Collectotrichum gleosporidose* OGC1 (21.4%); *Alternaria brassiceae* OCA3 (33.33%); *Rhizoctonia solani* MTCC 4633(35.7%) *and Phytophthora capsici* (36.6%) (Plate 4).

Plate 4: 9P showing antagonism against the four phytopathogens.

Table 2: Detection of other hydrolytic enzymes and PGPR traits by the chitinase positive isolates

Cultures	Hydrolytic enzymes					PGPR traits			
	Chitinase	Cellulase	Glucanase	Protease	Lipase	IAA	Sid	PO_4 Soln	HCN
9p	+	+	+	+	+	+	+	-	-
8p	+	+	-	+	-	+	-	-	-
1t4m	+	+	-	+	-	+	+	-	-
11p	+	+	-	+	-	-	-	-	-
13b	-	+	-	+	-	-	-	-	-
13a	+	+	-	+	-	-	-	-	-
2	+	+	-	+	-	ND	ND	ND	ND

ND-NOT DETERMINED

Plate 2: Detection of hydrolytic enzymes by the different isolates

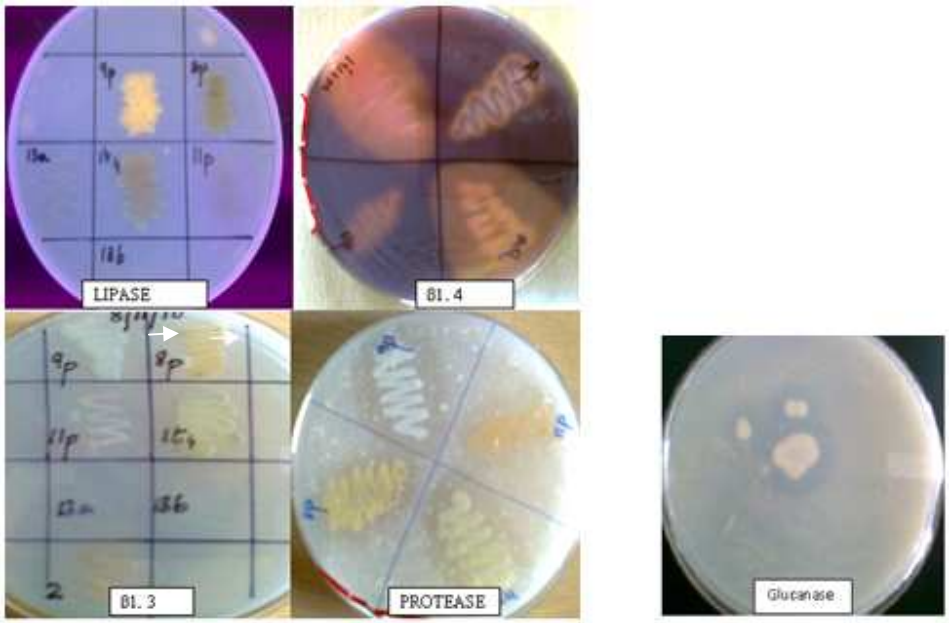
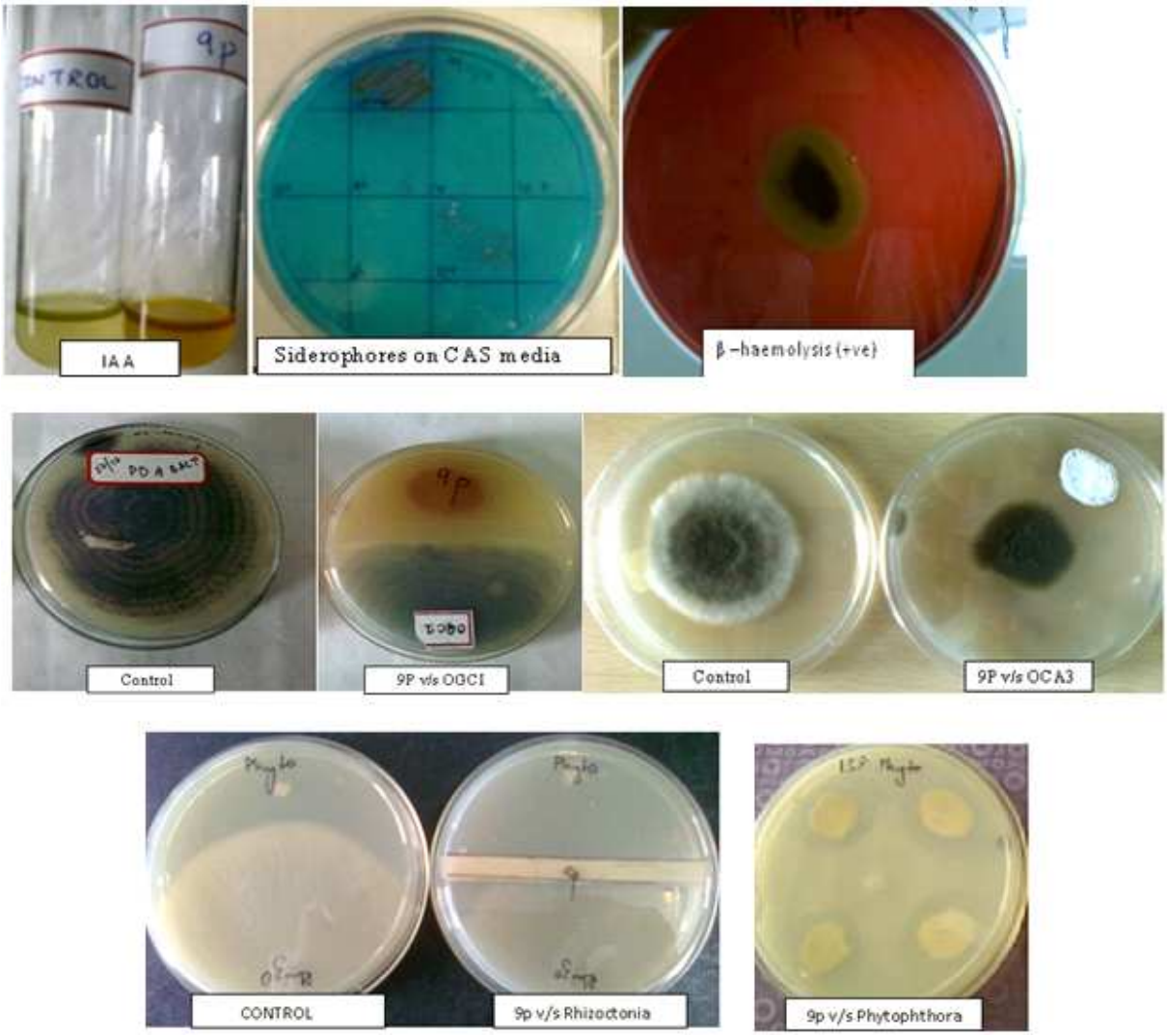


Plate 3: Results for IAA, siderophore and biosiurfactant production by Isolate 9p



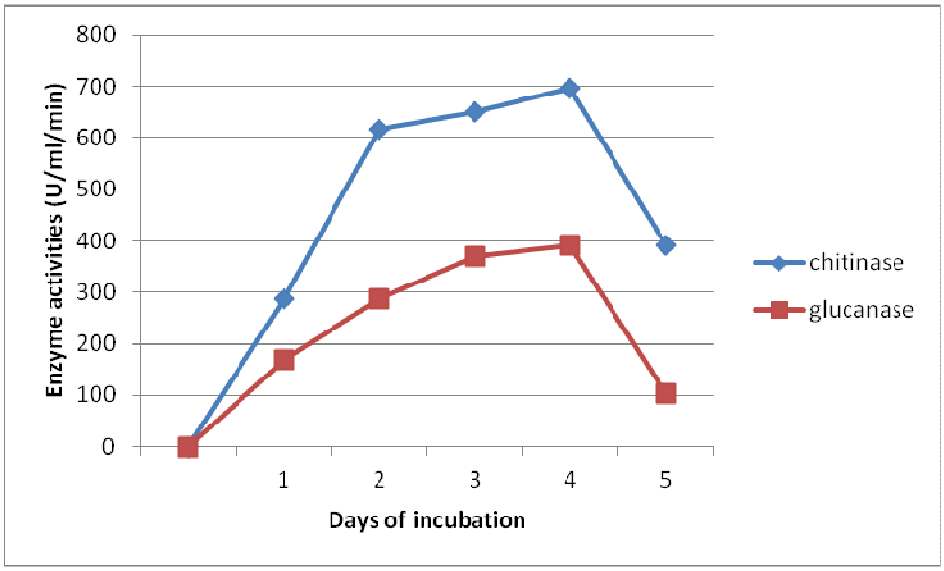
Actinomycete-fungus antagonism has been demonstrated for a wide variety of plant pathogens. Moreover, actinomycetes of the genus *Streptomyces* have been used to commercially control plant diseases. For example, *Streptomyces* sp. strain 5406 has been used in China for more than 30 years now to protect cotton crops against soil-borne pathogens [40]. More recently, Kemira Oy has developed a biofungicide that contains living *Streptomyces griseoviridis* cells to protect crops against *Fusarium* and *Alternaria* infections [41]. Antagonistic phenomena against fungi can be explained by several mechanisms, including antibiosis and parasitism. In some cases, hydrolytic enzymes such as chitinases [42], and other enzymes such as glucanases or proteases, may act against the fungal cell-wall, antibiotic production also being probably involved [43]. Based on the colony morphology, pigments, microscopic and i6srDNA studies, 9p was identified as *Streptomyces* sp (data not shown).

Induction of hydrolytic enzymes by isolate 9p

It has been reported that antifungal mechanism of antagonists has been attributed to the action of hydrolytic enzymes such as chitinase, β-1, 3-glucanase, chitosanase, and protease. The level of chitinase was sharply increased during the exponential phase and dramatically declined when the cells entered the stationary phase (Figure 1). The strain 9p produced relatively high levels of chitinase (15.4 U/mL) at day 4 of the incubation period. Meanwhile, the highest

level of β -1, 3-glucanase (8.7 U/mL) was also found at day 4 of the incubation period and subsequently decreased slightly during the stationary phase (Figure 1).

Figure 1. Chitinase and Glucanase enzyme profile of isolate 9p



It has been reported previously that actinomycetes produce extracellular β-1, 3- and β-1,4-glucanases [44,45]. As stated in many previous reports, the production of chitinase and β -1,3-glucanase enzymes by *actinomycetes* was related to fungal growth inhibition and the biological control of fungal pathogens was possible because of the ability of *actinomycetes* to degrade fungal cell walls [4]. It should be noted that one of the possible antifungal mechanisms of the *Streptomyces* strain 9p, in this study, may be associated with the production of extracellular chitinase and β -1, 3-glucanase enzymes.

In general, no defined medium has been established for chitinase production from different microorganisms. Each organism has its own requirement of special conditions for maximum enzyme production [46]. Joo [2005] described the stimulatory effect of starch and glucose in *Streptomyces halstedii* [47]. Gursharan *et al*., 2008 [48] also reported the stimulatory effect of starch, pectin, laminarin, and β-glucan on chitinase production by *Streptomyces lydicus* WYEC108.

Culture medium is a key factor for the growth as well as metabolites production by microorganisms. Among the various carbon sources tested in the ISP media, all carbon amendments supported induction of both chitinase and glucanase. Of the carbon supplements tested, ISP with CMC supported the highest production of both chitinase (8.7U/ml) and glucanase (9.2U/ml) on day 4. Even the autoclaved mycelia of *Colletotrichum gleosporoides* (OGC1) and *Alternaria brassicae* (OCA3) supported appreciable levels of both the enzymes (Figure 2). *Enterobacter* sp. NRG-4 produced a high level of chitinase when grown in the presence of fungal cell wall of *Candida albicans* and *Fusarium maliniforme* [46]. The effectiveness of *C.gloesporoides* and *A.brassicae* walls as glucanase inducers is in agreement with Tanaka's and earlier observation on the stimulation of β-1,3 glucanase synthesis in *Bacillus circuluns* by walls of baker's yeast [49]. Lilley and Bull (1974) also reported similar findings with *Aspergillus nidulans* and *Neurospora crassa* walls as glucanase inducers [50].

Seed Bacterization

Although rhizobacteria may present unique challenges to our attempts to harness their beneficial attributes, the prospects for improved agriculture by the use of biocontrol- PGPR seem excellent. Advances in our understanding of the PGPR Systems responsible for plant growth improvement is a first logical step in opening the way to improving these bacterial strains through genetic engineering, and generating more interest in their development for widespread commercial use for both biocontrol and plant growth promotion. It is believed that actinomycetes are

among the most promising biocontrol- PGPR in need of study in future research [51]. PGPR can affect plant growth in two general ways, either directly or indirectly.

Figure 2. Enzyme profile of isolate 9p on day 4 using various substrates

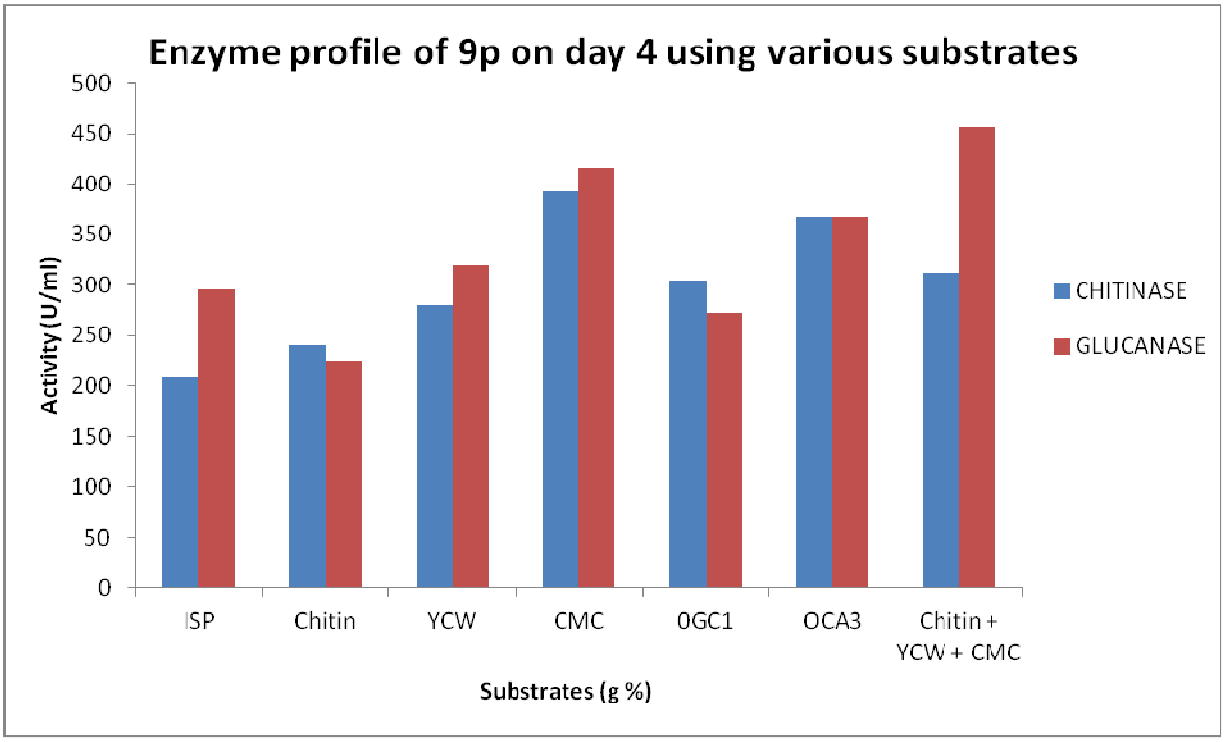
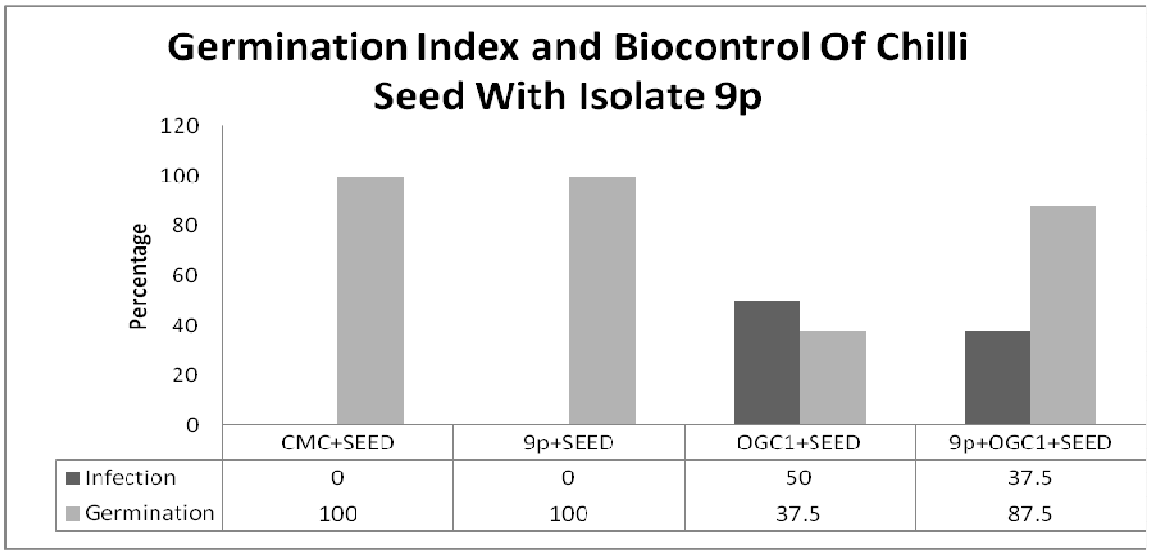


Figure 3. Seed bacterization results with respect to germination index and biocontrol efficiency of 9p on chilli seeds



Indirect promotion occurs when PGPR lessen or prevent the harmful effects of one or more deleterious microorganisms. This is chiefly attained through biocontrol, or the antagonism of soil plant pathogens. Specifically, colonization or the biosynthesis of antibiotics [52] and other secondary metabolites can prevent pathogen invasion

and establishment. Direct promotion of plant growth by PGPR occurs when the plant is supplied with a compound that is synthesized by the bacteria, or when PGPR otherwise facilitates plant uptake of soil nutrients. Possibilities include nitrogen fixation, siderophore synthesis, phytohormone synthesis, and solubilization of minerals to make them available for plant uptake and use [53].

Chilli seeds were coated with the isolate [9p] with/ without the *Collectotrichum* spores and was tested for the growth promotion, biocontrol efficiency and germination properties of the isolate. Treatment of the chilli seeds with isolate 9p showed 100% germination index similar to untreated (Figure 3). The treatment of the seed with co-inoculation of the pathogen with 9p showed 75% reduction in seed mortality by the treatment as compared to the seed treated with pathogen alone. This treatment also showed 87.5% germination index suggesting both the biocontrol and PGPR aspect of the bacteria. Inoculation of 9p with seeds showed the presence of more lateral roots as compared to uninoculated control seeds (Figure 3). There are reports of antagonism of chilli phytopathogen *Pyhtium debaryanum* by soil fungi from chilli rhizosphere [54].

CONCLUSION

Many investigators have suggested the rhizospheric bacteria *Pseudomonas* spp. as very interesting sources for the identification of antimicrobial compounds and their practical use as biopesticides [28, 55]. Thus, it is evident from the present study that actinomycetes 9p under investigation is also capable of producing plant growth promoting substances and mediate antagonism against phytopathogens through mixed type mechanism of mycolytic enzymes. Hence actinomycete 9p, a *Streptomyces* sp. is a potential candidate for the development of bioinoculants for crop plants.

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