Antiphytopathogenic activity of bacterial protein of a marine *Corynebacterium* sp. isolated from Mandapam, Gulf of Mannar

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ABSTRACT

The present study was carried out to validate the sensitivity and precision of an *in vitro* assay for evaluating the efficiency against antiphytopathogenic fungi by bacterial species *viz*. *Lactobacillus* sp, *Corynebacterium* sp, *and Aeromonas* sp. isolated from Mandapam, Gulf of Mannar. The bacterial protein was extracted from the isolated bacteria and screened against ten phytopathogenic fungi. Among the three bacterial isolates only *Corynebacterium* sp. exhibited antagonism against phytopathogenic fungi. The minimum inhibition was observed with *Aspergillus niger* (2 mm) and the maximum inhibition against *Alternaria alternata* (20mm). The optimized growth parameters of *Corynebacterium* sp. was carried out for mass scale production. The antiphytopathogenic protein was partially purified by subjecting to ammonium sulphate precipitation followed by TLC and gel electrophoresis. SDS – PAGE analysis reported two protein bands corresponding to 34 kDa and 71 kDa.

Key words: Anti-phytopathogenic activity, bacterial protein, *Corynebacterium* sp., optimization, purification

INTRODUCTION

The surfaces of aerial plant parts provide a habitat for epiphytic micro-organisms, many of which also influence the growth of pathogens. Bacteria are generally the predominant initial inhabitants of newly expanded leaves, while yeasts and filamentous fungi dominate later in the growing season (Kinkel *et al*., 1987). A large body of information has been accumulated regarding antagonism between bacteria and fungi on the leaf surface and its possible role in the biological control of pathogenic fungi (Gowdu and Balasubramanian, 1988). With an increasing misuse of antibiotics, the serious problem of antibiotic resistance is coming up very fast. Therefore, intensive search for new antibiotic is going worldwide (Katz and Demain, 1977; Leisinger and Margraff, 1979; Emmert *et al*., 2004). To make the production of antibiotic feasible, it is necessary to develop the optimum production conditions. Several researchers have contributed considerably in this field (El-Banna, 1989; Akihiro *et al.*, 1993; El-Banna and Winkelmann, 1998).

Coryneform (irregular) bacteria include various groups of aerobic non-spore forming, non-acid-fast, non-motile irregularly shaped, gram-positive rods which are very diverse not only morphologically but also metabolically and structurally (Funke *et al.*, 1997). The majority of coryneform bacteria are environmental residents and or normal flora, and they are isolated frequently in clinical laboratories (Tang *et al.*, 2000). Species of the genus *Corynebacterium* can be isolated from soil, air, water, blood, and even human skin (Funke *et al.*, 1997 and Janda, 1998). Corynebacteria can infect plants, animals and rarely humans (Collins and Cummins, 1984; Barthold and Brownstein, 1988). Several species of Corynebacteria were involved in the process of oil and oil-related products removal from the environment (Bicca *et al.*, 1999).

The geneus *Corynebacterium* consists of gram positive, strictly aerobic, polarly flagellated rods. They are aggressive colonizers of plants and have a broad spectrum antagonistic activity against plant pathogens such as antibiosis siderophores production and nutrition (or) site competition (Rosales *et al*., 1995). *Corynebacterium* species has been suggested as a good candidate for use as seed inoculants and as root dip for biological control of soil-borne plant pathogens. *Corynebacterium* sp. has the ability to grow at 4°C and hydrolyse gelatin. This characteristic explains its frequent occurrence in spoilage of refrigerated food. The main property that conspires against its becoming important opportunistic pathogen is the inability to grow at body temperature. It is rarely pathogenic for humans, even though they have been found associated with empyema, urinary tract infections and septicemia. Some bacteria have been recognized as antagonist of plant fungal pathogens and antibiotic producers (O'Sullivan and O'Gara, 1992). Antagonistic activity of *Corynebacteriun* spp. was observed in the soil and it had been recognized as a major factor in the suppression of many phytopathogens.

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The possibility for these bacteria, which show antifungal activity and fungal growth modulation activities, might be incorporated in the developing ascocarphy means of their preferential adhesion to tuber mycelium (Sbrana *et al*., 2000). The present study was to examine the antifungal activity of *Corynebacterium* against different plant pathogenic fungi.

Rhizosphere bacteria belonging to the fluorescent pseudomonads are receiving increasing attention for the protection of plants against soil-borne fungal pathogens (Afsharmanesh *et al.*, 2006).

Stenocarpella maydis and *Stenocarpella macrospora* are the causal agents of ear rot in corn, which is one of the most destructive diseases in this crop worldwide. From the cultures of *Bacillus subtilis*, *Pseudomonas* spp., *Pseudomonas fluorescens*, and *Pantoea agglomerans*, extracellular filtrates were obtained and assayed to determine antifungal activity. The best filtrates were obtained in the stationary phase of *B. subtilis* cultures that were stable to the temperature and extreme pH values (Ivan Petatan-Sagahon *et al.*, 2011).

Specific strains of fluorescent Pseudomonas spp. inhabit the environment surrounding plant roots and some even the root interior. Introducing such bacterial strains to plant roots can lead to increased plant growth, usually due to suppression of plant pathogenic microorganisms (Mercado-Blanco and Bakker, 2007).

Antimicrobial substances are mainly produced by bacteria and lower fungi, and have many roles in the treatment of most infectious diseases, food preservation, animal nutrition and plant protection (Berdy, 1974). In addition, many antimicrobial substances have been used in research executed at the molecular biology level (Lancini and Parenti, 1982). The objective of the present investigation was to screen new bacterial isolates for their ability to produce antimicrobial substances.

MATERIALS AND METHODS

Isolation of Bacteria

Sediment samples were collected from Mandapam (Lat 8° 35' 15'' N; Long 78° 08' 25'' E) situated in Gulf of Mannar region. To isolate the marine bacteria, 1g of marine sediment sample was suspended in 99ml of sterile 50% aged sea water. Samples were serially diluted and 0.1ml of sample was spread plate on Zobell Marine Agar (ZMA) and incubated at 35^0C for 48 hrs. Colonies were counted and the results were expressed as CFU/g. The various fungi used in the present study were proven pathogens obtained from CAS Botany Chennai and Tamil Nadu Agricultural University, Coimbatore.

Biochemical Characterization of *Corynebacterium* sp.

The serial dilution agar plate method was used to isolate *Corynebacterium* species on Zobel Marine Agar medium. The Pigmentation and biochemical reactions were determined as described in Bergey's manual of Determinative Bacteriology (Holt *et al.,* 1994). The *Corynebacterium* sp. Was a Gram Positive, non motile, non spore forming rod. It showed the positive reactions in Catalase, Starch hydrolysis, Glucose, and Maltose fermentation test. Negative in IMViC tests, Gelatin hydrolysis. Fermentation of Arabinose, Xylose, Lactose, Sucrose, Raffinose, Galactose, and Manitol and in Oxidase test.

Antagonistic activity

Antagonistic activity of marine bacterial isolates was tested as described by Geels and Schipper (1983). Initial screening for *in vitro* antagonistic activity was tested against fungal strains on PDA agar plates. The strain which higher activity showed was selected. The biochemical analysis for the potential strain was carried out according to Buchanan *et al.*, (1974).

Culture filtrate activity

The culture filtrate activity of marine bacterial isolates was tested against plant pathogenic fungi. After swabbing the pathogens on the plates, 0.1 ml of cell free culture broth (log phase) of *Corynebacterium* was centrifuged at 10,000 rpm for 20 min., and poured into the well of plates and incubated at 30^0C for 3-4 days. The bacterial culture filtrate inhibiting the growth of pathogen around the well after incubation was assessed by the inhibition zone around the well and results were recorded.

Optimization

Different combinations and sequences of process conditions are needed to be investigated to determine the growth conditions which produce the maximum biomass with the physiological state best constituted. Method of changing one independent variable at a time was followed and variables like pH (6, 7, 8, 9, 10 and 11), temperature (25°C, 30°C, 35°C and 40°C), salinity (0.5%, 1%, 1.5%, 2% and 2.5%) and substrates (glucose, sucrose, fructose, lactose, starch, glycerol and maltose) were tried. The nitrogen sources such as ammonium nitrate, potassium nitrate and ammonium sulphate were checked after 54 hrs incubation. Absorbance was measured at 600nm at an interval of 6 hrs.

Protein Extraction and Estimation

When optimum conditions were kept, maximum biomass as well as more extracellular protein was produced. The protein

was extracted by 65% of ammonium sulfate. The protein concentration of the sample was determined by Lowry's method (Lowry *et al*., 1951) using bovine serum albumin as standard.

Ammonium sulphate precipitation and dialysis

The shake flasks kept for mass scale production were taken after 36 hrs and centrifuged at 15,000 rpm for 10 min. To the supernatant, the amount of ammonium sulphate required to give 65% saturation was added slowly with stirring. Dialysis was followed in a tubular cellulose membrane against phosphate buffer for 24 hrs at 4°C. The partially purified protein was lyophilized in a Vertis lyophilizer and kept for further analysis (Sambrook *et al.*, 2001).

RESULTS

In the present study marine sediment samples of Mandapam in Gulf of Mannar area were plated on Zobell marine agar and the density was found to be $1.3x10^7$ CFU/g.

The present study was carried out to validate the sensitivity and precision of an *in vitro* assay for evaluating the efficiency of inhibition of phytopathogenic fungi by bacterial species *viz*., *Lactobacillus* sp., *Corynebacterium* sp., *and Aeromonas* sp. isolated from Mandapam, Gulf of Mannar. Among the three bacterial isolates the *Corynebacterium* sp. only exhibited antagonism against phytopathogenic fungi. The culture filtrates as well as concentrated protein showed antifungal activity against all the ten fungai tested Against *Alternaria alternata* maximum zone (20 mm) was observed with protein concentrate. It was followed by *Sclerotium roysii* and *Rhizoctonia solani* (18 mm), *Colletotrichum musae* (10 mm), *Colletotrichum capsici* (8 mm), *Colletotrichum lamella* (5 mm), *Gleosporium gleosporioide* (4 mm), *Phytophthora infestans*- FMC 42 (3 mm), *Aspergillus niger* (2 mm) and *Macrophomina phaseolina* (no activity).

The isolate (SBS-6) was identified by the use of biochemical characters and Bergey's Manual of Systemic Bacteriology. Strain SBS-6 is non-motile, non-spore forming, gram-positive, aerobic rod shaped bacterium and was identified as *Corynebacterium* sp.

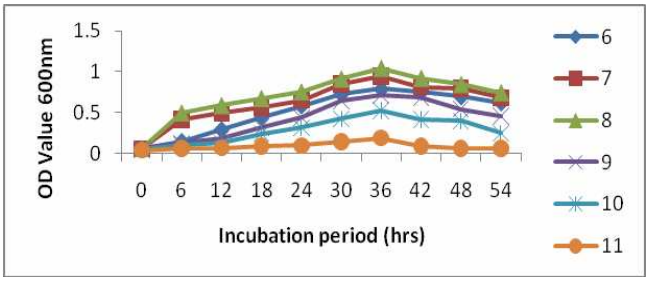

Fig. 1. Effect of pH on growth of *Corynebacterium* SBS-6.

Optimization conditions for growth

Parameters like pH (8) (Figure 1), temperature (35°C) (Figure 2), salinity (1%) (Figure 3), carbon sources (glycerol) (Figure 4) and nitrogen sources (yeast extract) (Figure 5) were optimized for growth as protein production found to be in direct SBS-6 strain relationship in this *Corynebacterium* sp.

The optimized growth parameters of *Corynebacterium* sp. was carried out by mass scale fermentation. The antiphytopathogenic protein was partially purified by subjecting to ammonium sulphate precipitation and it was confirmed as protein by TLC and SDS–PAGE analysis showed that two protein bands with antifungal activity were present corresponding to 34 kDa and 71 kDa (Figure 6).

DISCUSSION

Biological control may be an alternative to chemicals in the control of some pathogenic fungi, reducing environmental pollution (Handelsman *et al*., 1990). Certain rhizosphere bacteria including *Pseudomonas* sp. and *Corynebacterium* sp. were used to control different plant fungal diseases like *Pythium* damping-off and some root rot fungi (Parke *et al*.,

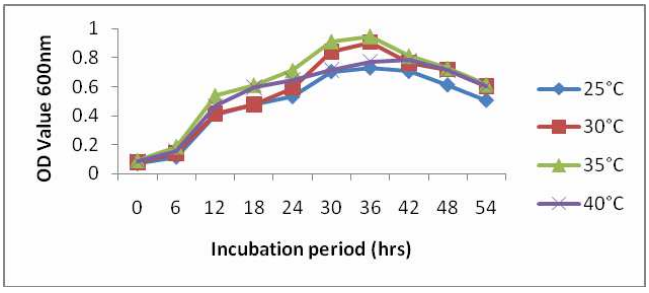

Figure 2. Effect of temperature on growth of *Corynebacterium* SBS-6.

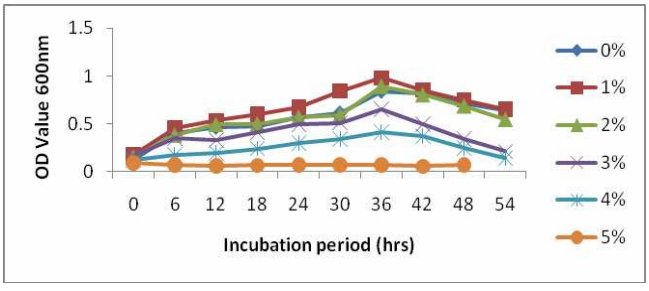

Figure 3. Effect of salinity on growth of *Corynebacterium* SBS-6.

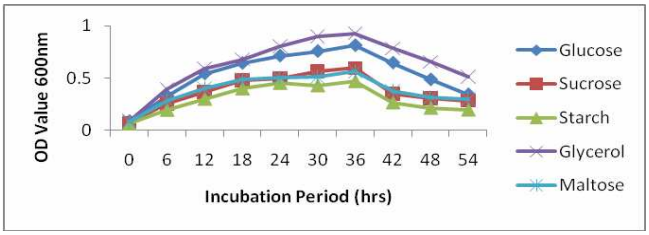

Figure 4. Effect of carbon sources on growth *Corynebacterium* SBS-6.

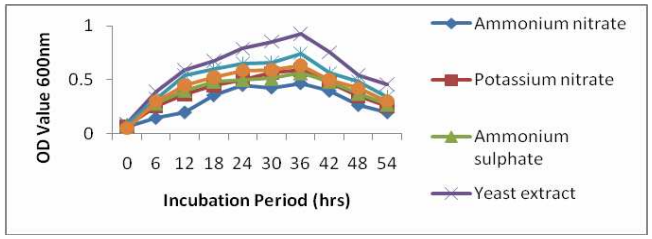

Figure 5. Effect of nitrogen sources on growth of *Corynebacterium* SBS-6.

1991). Others like *Bacillus subtilis* that exhibits insecticidal, antifungal and antibacterial activities were used to control *Rhizoctonia solani* damping-off (Asaka and Shoda, 1996), and bean leaf rust caused by *Uromyces phaseoli* (Baker *et al*., 1983). Some bacteria species were found to show antimicrobial activities (Gebreel, 2008) and effectively control brown rot of rice (Islam and Nandi, 1983).

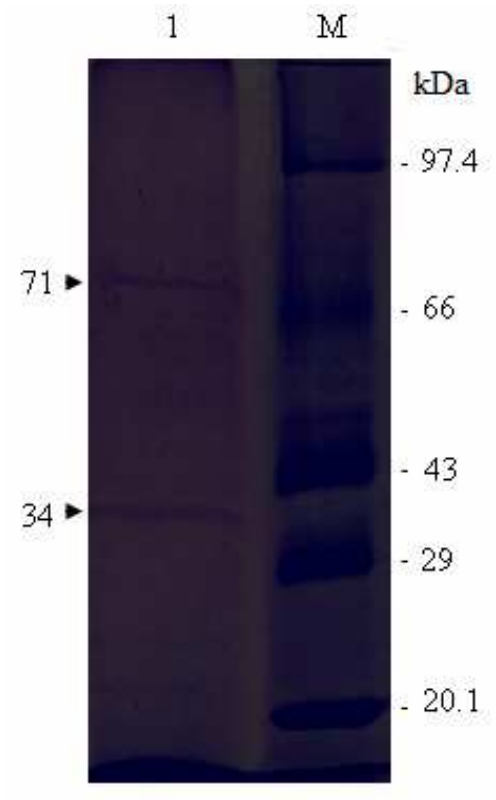

Figure 6. Protein profile of bioactive compound on SDS-PAGE Gel

Lane M: Standard Protein Molecular Weight Marker and Lane1: Cell free extract with bioactive compound

In the present study bacterial strains were isolated and tested for their antagonistic ability against phytopathogenic fungi. The selected antagonistic *Corynebacterium* sp. showed a very broad range of antagonistic activity against all the phytopathogenic fungi tested. The minimum inhibition was seen against *Aspergillus niger* (2 mm) and the maximum inhibition was found with *Alternaria alternata* (20 mm).

Bacteria-fungal pathogen interactions are having an increased interest in the area of biocontrol. Many of the antifungal interactions involved *Corynebacterium* sp. This is proved in the present study where *Corynebacterium* sp. exhibited inhibitory activity against proven plant pathogens of crops.

The strain SBS-6 appeared to have the strongest activity against all the fungi tested. Further work on mode of action is in progress. The mechanisms of disease reduction may involve siderosphore mediated competition, which result in the exclusion of fungal pathogens by a reduction in the availability of iron for the survival of pathogens (Schippers *et al.*, 1987). Fluorescent bacteria are known to have a significant role in the suppression of fungal pathogens by the production of siderosphores (Hamdram *et al.*, 1991).

Effect of different temperatures (25, 30, 35, and 40°C) on the growth was studied by inoculating the test strain in the medium. Antimicrobial activity was measured in terms of zone of inhibition (data not shown). Best activity was shown at temperature 35°C.

When effect of pH on growth of *Corynebacterium* sp. SBS-6 was studied, it was evident from the results that SBS-6 showed biomass at pH 8, and when tested against *Alternaria alternata* it showed maximum inhibition. The pH of media between 7-8 had maximum antimicrobial production, by *Bacillus subtilis* MZ-7 (Muazz *et al.*, 2007). Best activities against *Micrococcus luteus* were shown at pH 8, while at pH 6 and 7, a decrease in activity was observed. At pH 9, second best activities were observed (Awais *et al*., 2008).

Effect of different carbon sources on the growth was studied by inoculating the test strain in the medium. The maximum growth was observed in glycerol. The carbon source needed

for maximal yield of the antibiotic production seems to be different among bacterial strains. Glycerol supported better antibiotic production by *Streptomyces hygroscopicus* D 1.5 (Bhattachryya *et al*., 1998). Glucose was also reported as the most suitable carbon source for maximum phenazine production by *Pseudomonas fluorescens* 2-79 (Slininger and Shea-wilbur, 1995).

Among nitrogen sources tested, yeast extract showed maximum growth. Preference of nitrogen source as well as its concentration seems to vary according to the strain involved. In *Streptomyces hygroscopicus* ammonium succinate markedly increase the rate of antibiotic production. Genesheva *et al.* (2005) who worked on this actinomycete opined that depending on the biosynthetic pathway involved, nitrogen source may vary and it influences the antibiotic production. Undoubtedly the present study along with other studies reviewed through literature indicated that complicated interactions occur in rhizosphere between the plant and the microbes. The *Corynebacterium* strain isolated in the study seemed to be highly potential in controlling fungal pathogens infecting commercial crops. Further study in this strain may result in the development of a potential biocide.

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