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SCREENING AND INVITRO CHARACTERIZATION OF SIDEPHORE PRODUCING *PSEUDOMONAS FLUORESCENS*

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ABSTRACT

Pseudomonas fluorescens is a common gram negative, rod shaped bacterium. *Pseudomonas fluorescens* produce nearly eight secondary metabolites. Pseudomonas species have been widely studied as biological agents (BCAs) and it is alternative to the application of chemical bactericides. The objective of present study was to screening of the isolate which showed varied levels of PGPR traits – siderophore, HCN and IAA. The isolate K was identified as *Pseudomonas fluorescens* by biochemical tests. Concurrent production of siderophore, IAA, HCN, NH_3 and catalase suggests the isolate K possesses plant growth promotion and broad spectrum biocontrol potential.

KEYWORDS: *Pseudomonas fluorescens*, siderophore, IAA, HCN**,** biocontrol agent.

1. INTRODUCTION

Many bacteria are present in the rhizosphere have antagonistic action, which safeguard plants from pathogens and stimulate growth.[1] Biological control of plant diseases using antagonistic microorganisms offers a highly effective, economical and environmental friendly alternative to the use of synthetic pesticides.[2] The mode of action of the antagonistic organisms against various soil-borne plant pathogenic fungi, include biosynthesis of antibiotics, production of hydrolytic enzymes[3], production siderophore and competition for substrates. Successful bacterial antagonists often show a synergistic combination of mechanisms responsible for a successful antifungal interaction. Fluorescent pseudomonades

(*Pseudomonas fluorescens, P. aeruginosa, P. putida*) and closely related species are important antagonistic bacteria present in soil.[4] Some of the fluorescent pseudomonades have currently received world-wide attention due to the production of a wide range of antifungal compounds viz., fluorescent pigments, siderophores, volatile compounds such as hydrocyanic acid (HCN) and antibiotics. *Pseudomonas fluorescens* is a common gram negative, rod shaped bacterium. *Pseudomonas fluorescens* produce nearly eight secondary metabolites. Pseudomonas species have been widely studied as biological agents (BCAs) and it is alternative to the application of chemical bactericides. The main objectives of the present study screening and invitro characterization of sidephore producing *Pseudomonas fluorescens*.

2. MATERIALS AND METHODS

Isolation of rhizospheric bacteria

Bacteria were isolated from solanaceae rhizosperic soils like potato, capsicum and chilli grown in Pulivendula, Andhra Pradesh, India by soil dilution method. The different isolates obtained on nutrient agar were screened for protease production on potato dextrose agar plates according to.[5] The isolate was routinely maintained on nutrient agar slants at 4˚C.

Characterization of Pseudomonas isolates

Identification of the selected Pseudomonas was carried out by Biochemical test (Methyl Red Test, Catalase Test and Citrate test as per standard procedures).

Detection of Hydrolytic Enzymes

Protease activity according to Berg et al.[6], and cellulolytic activity on microcrystalline cellulose-containing plates as described by Teather et al.[7] Lipase was detected qualitatively by fluorescence caused by the fatty acid released due to the action of lipase on olive oil, based on interaction of Rhodamine B with fatty acid released during the enzyme hydrolysis of olive oil.[8]

Detection of secondary metabolites

Siderophore Production Siderophore production was tested by growing Pseudomonas sp. in the universal siderophore detection medium CAS agar.[9]

HCN Production and catalase

Hydrogen cyanide production was assayed by the method suggested by Castric (1977).[10] For catalase detection, bacterial cultures were grown in a nutrient agar medium for 18-24 h at 36±2 °C. The cultures were mixed with appropriate amount of H_2O_2 on a glass slide to observe the evolution of oxygen.

IAA production

Bacterial cultures were grown for 48 h on their respective media at 36±2°C. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). Development of pink colour indicates IAA production.[11]

Detection of ammonia

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48–72 h at 36±2 °C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow color was a positive test for ammonia production.[12]

3. RESULTS AND DISCUSSION

Isolation of proteolytic positive bacteria

Detection of protein-degrading bacteria from natural sources such as rhizosphere soil is useful in the isolation of bacteria that produce antibacterial or other novel compounds. In this study, 18 bacterial isolates were isolated from the rhizosphere of solanaceae family namely potato, capsicum, chilly and screened for the production of protease enzyme. Total of 15 isolates (75.55%) were the most potent proteolytic bacterial species. Of these, 5 isolate showing zone size > 5 mm were chosen for further studies. Screening of proteolytic bacteria isolates was carried out by spread inocula of each colony on plates containing a potato dextrose agar as a sole carbon and energy source. The proteolytic degrading organism formed colonies of 1-2 mm in diameter, surrounded by clear zones indicating protease activity.

Biochemical Characterization of *Pseudomonas fluorescens*

Methyl red test

On addition of drops of methyl red indicator to the incubated Methyl red broth, then the broth changes to yellow colour, which indicates negative result (Fig: 3.1).

Fig. 3.1: Methyl Red Test result.

As this organism is not having any ability to produce acids as end products from glucose fermentation, so it shows the colour yellow instead of red by adding methyl red indicator.

Citrate test

The turbidity in the Koser's medium and Change of colour(from light gren-blue) in simon's citrate agar was observed with indicates the utilization of citrate as a carbon source by microorganism (Fig.3.2).

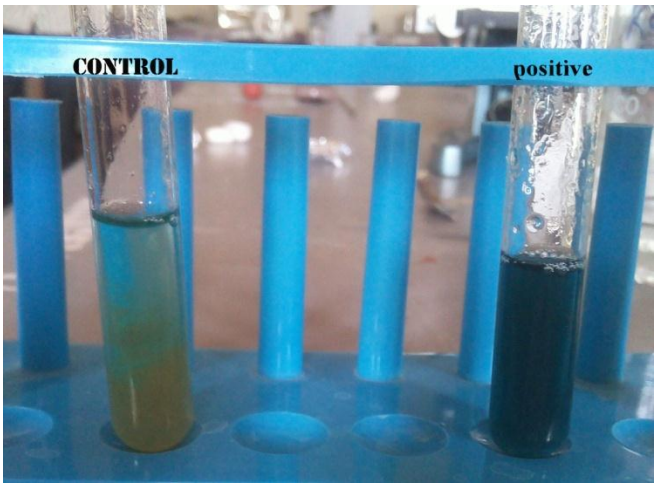

Fig 3.2: Citrate Test result.

Change of colour from light green to blue shows the ability of the organism to utilize citrate as carbon and energy source.

Catalase test

On addition of Hydrogen peroxide to culture enormous amount of bubble formation was observed which shows the hydrolysis of hydrogen peroxide to (Fig.3.3).

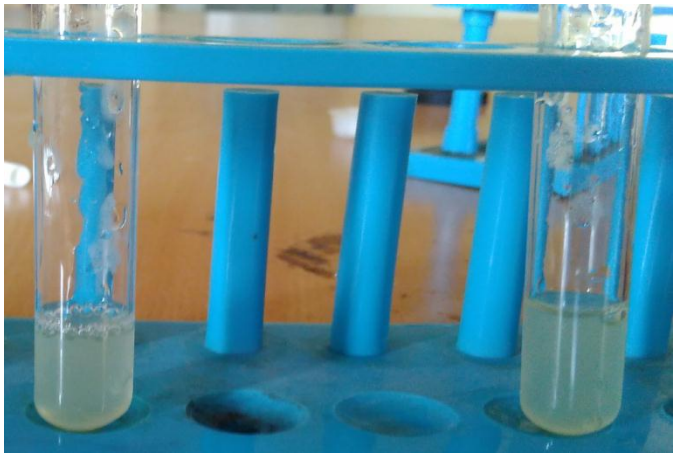

Fig 3.3: Catalase Test result.

Due to the production of catalase enzyme by the organism help in breakdown of hydrogen peroxide to water and oxygen. So the bubbles are formed in this test.

Production of siderophores

Siderophores production was optimum is succinate medium. The colour change from colourless Pale/light yellow colour and change in pH of the medium indicates production after 24 hours incubation in Orbital shaking incubator at 120 rpm 24 h.

The siderophores are collected by centrifuging the medium Supernatant was collected and stored. The colour change from colourless to golden yellow shows the production of siderophores from *Pseudomonas fluorescens*. Siderophore could be detected only after 12 h, a slight yellowing of the medium indicated the beginning of siderophoregenesis, which was found increasing and reaches maximum level during 24 h.

Detection of siderophores

Chrome Azurol Assay

CAS reagent was added to the supernatant which is isolated from succinate medium. Formation of blue colour due to presence of insoluble Iron complex, after adding Shuttle solution it turns to orange colour in few minutes (Fig.3.4).

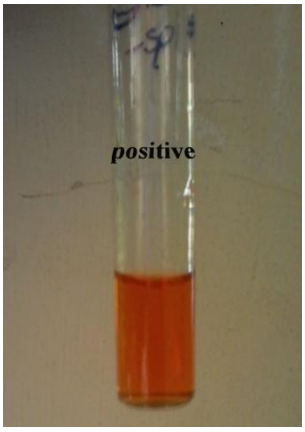

Fig 3.4: Chrome Azurol sulphate liquid assay.

Characterization of Siderophores

Hydroxamate type siderophores

The formation of orange colour complex indicates production of hvdroxamate siderophores (Fig.3.5).

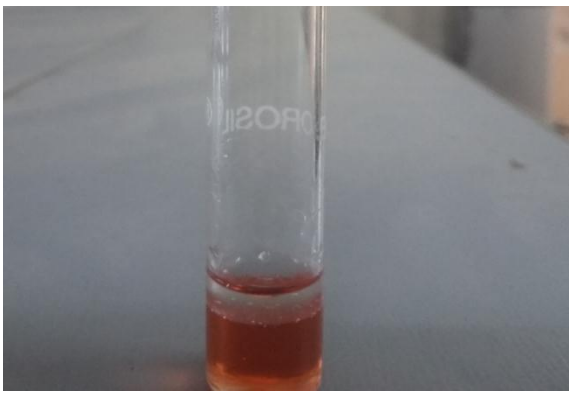

Fig 3.5: Assay of Hydroxamate Siderophores.

Due to the addition of ferric chloride solution to the supernatant it hydrolyses the amino acids present in this siderophore and change the colour of the solution to orange.

Catecholate type Siderophores

The formation of wine red color complex indicates production of catecholate siderophores (Fig.3.6).

Fig 3.6: Assay of catechoate siderophores.

Different enzymes are involved in the synthesis of this siderophore and by the addition of ferric chloride to the solution containing this siderophore helps in hydrolyses of amino acids and changes the colour to wine red.

Detection of Hydrogen cyanide

The Whatmann No.1filter paper colour changed from orange to red colour due to release of hydrocyanic acid (Fig.3.7).

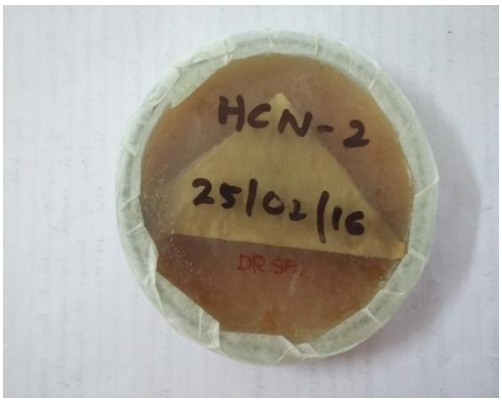

Fig 3.7: Detection of Hydrogen cyanide.

In present work, *Pseudomonas flurescens* isolate K were positive for the HCN production, which acts as inducer of plant resistance. It is secondary metabolite produced by gram negative bacteria *Pseudomonas flurescens*, and is postulated to play important role in biological control of pathogens .Its production is determined by the change of filter paper colour from orange to red due to release of hydrocyanic acid by organism.

Detection of Indole acetic acid

By adding orthophosphoric acid: Solawaski's reagent in 1:2 ratio to the 2ml of supernatant. Pink colour was developed indicates presence of indole acetic acid (Fig. 3.8).

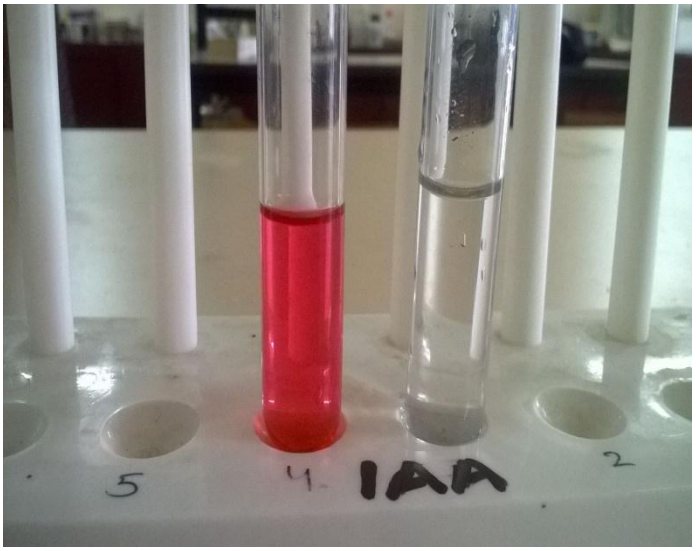

Fig 3.8: Detection of Indole Acetic Acid.

IAA is one of the most important phytohormone and function as important signal molecule in the regulation of plant development during the onset of symbiosis in legumes. The ability of bacteria to produce IAA in the rhizosphere depends on the availability of precursors and uptake of microbial IAA by plants.

The overall biochemical test performed for *Pseudomonas fluorescens K* were shown in Table 3.1.

Table 3.1: Over all biochemical test performed for *Pseudomonas fluorescens K.*

S.No	Biochemical test	Pseudomonas fluorescens
1	Methyl red test	+
2	Citrate test	+
3	Catalase test	+
4	Hydrogen cyanide test	+
5	Indole acetic acid test	+

Determination of PGPR traits & In vitro characterizations of the biocontrol mechanism of isolate K

Many species and specific strains of bacteria residing in rhizosphere have been shown to possess plant growth promoting traits and hence they are collectively designated as plant

growth promoting rhizobacteria (PGPR).[13] PGPR enhance plant productivity by a range of direct/ indirect mechanisms. These beneficial effects of PGPR can be either direct or indirect. Direct promotion of growth by PGPR occurs when the rhizobacteria produce metabolites that promote plant growth such as auxins[14], cytokinins[15] and gibberellins.[16,17] Indirect growth promotion occurs through the elimination of pathogens by the production of cyanide[18] and siderophores. PGPR beneficial effects have been exploited in many areas including biofertilizers, microbial rhizoremediation and biopesticides.[19]

Table 3.2: Detection of PGPR traits of isolates.

Isolates	Siderophores	IAA	HCN Production
D	-	-	-
E	-	-	-
F	-	-	-
G	-	-	-
K	+	+	+

Among five strains, isolate K shown positive results for sidephore, IAA and HCN production. So these isolate K considered as potential isolate for the further studies for characterization of *Pseudomonas fluorescens* (Table 3.2).

CONCLUSION

The present study has identified as *Psuedomonas fluorescens* strain based on biochemical characterization producing hydroxamate and catechalate type of sidephores possessing multiple mechanism of broad spectrum antagonism which can be explored as one among the best biocontrol agent against phytopathogens.

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