

Original Research Article

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Isolation, Characterization of Bioactive Secondary Metabolites Producing *Pseudomonas* species from Soil of Marathwada Region, India

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ABSTRACT

The production of secondary metabolites from *Pseudomonas* species has been the most economical and biotechnological source for the discovery of new bioactive compounds. The aim of the present study was to isolate potent bioactive secondary metabolites producing *Pseudomonas* spp. strain from the agricultural soil of Marathwada region. There were 16 isolates obtained from different areas in Marathwada region and studied for the production of secondary metabolites and its antibacterial properties. These antimicrobial compounds were extracted and tested against some pathogenic microorganisms such as *Bacillus subtilis*, *Escherichia coli*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Proteus vulgaris* and *Klebsiella pneumoniae*. The results showed that among the isolated *Pseudomonas* spp. three of the isolates namely S3, S6, and S15 were found to be of potential antagonists against pathogenic organisms and thus providing the production of secondary metabolites which has the potent bioactive compounds to control the soil borne pathogens.

Keywords

Secondary Metabolites, *Pseudomonas* species, Bioactive compounds

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Introduction

The Agricultural soil of India is an extensively unexplored source for microorganisms with the potential to produce secondary metabolites of biological importance. The secondary metabolites from microorganisms play a vital role as lead compound in developing new drug and in chemotherapeutics (Samuilov, 2003). Due to their bioactive properties, secondary

metabolites have been traditionally mined from producing organisms for use in the pharmaceutical industry. Pharmacologically significant antibacterial secondary metabolites such as penicillin and vancomycin inhibit bacterial cell wall synthesis (Rai, *et al.*, 2003; Allen and Nicas, 2003) while tetracycline and erythromycin inhibit bacterial protein synthesis (Metcalf, *et al.*, 2002; Schlunzen, *et al.*, 2003). *Pseudomonas* spp. has gained

major attention in the agricultural industry because of its wide spread application in various biotechnological processes. An important ubiquitous member of this group, *Pseudomonas aeruginosa* is an opportunistic pathogen of plants and humans (Walker, *et al.*, 2004; de Bentzman and Plesiat, 2011). The conscious agricultural applications of *Pseudomonas aeruginosa* not only pose a threat to human health and environment but also raise relevant ecological issues such as evolution of multi resistant bacteria and pathogenicity. Hence, deliberate application of strains of this organism, or any other microorganism with such pathogenicity should be carried out with immense care, following bio safety regulations. Significantly smaller antimicrobial compounds produced by *Pseudomonas aeruginosa* are a diverse assemblage of heterocyclic compounds including the phenazines, quinolines and compounds containing a pyrrole moiety. The phenazines, produced by *Pseudomonas* spp. as well as *Streptomyces*, *Microbispora*, and *Sorangium* spp., are pigments demonstrating broad-spectrum antimicrobial activity (Gerber, 1973). The production of secondary metabolites from *Pseudomonas* spp. has been the most economical and biotechnological source for the discovery of new antibiotics.

It has already been leads to some important antimicrobial drugs like vancomycin, chloramphenicol and tetracycline. The development of new naturally occurring antimicrobial agents with novel mechanisms of action is an urgent medical need. Soil is an extensively exploited ecological niche; the inhabitants of soil produce several useful bioactive natural products, including clinically important antibiotics. The objective of the present study was isolation of *Pseudomonas aeruginosa* from the agricultural soil and its characterization production of bioactive compounds and evaluation of its growth inhibiting activities for sustainable agriculture.

Materials and Methods

Study area

The Marathwada region is the part of Maharashtra State of India. The study area was Jalna District of Marathwada region. The Jalna lies between 19.1 and 20.3 North Lat. 75.4 to 76.4 East Long. Jalna is significant for its unique ecological, geomorphological and biological profile.

Sample collection

Three soil samples were collected from Jalna District (Marathwada regions, India), for isolation of secondary metabolites producing microorganisms (bacteria). Soil samples (approx. 500 g) were collected by using clean, dry and sterile polythene bags. The site selection was done by taking care of the point where widely varying characteristics such as, the organic matter, moisture content, particle size and color of soil, are possible so as to avoid contamination as far as possible. Samples were stored in ice boxes and transported to the laboratory where they were kept in a refrigerator at 4⁰C until analysis (Marathe, *et al.*, 2015).

Isolation and characterization of bacteria

For the enrichment, one gram of each soil sample was serially diluted in physiological saline (0.85%, NaCl, w/v) containing quartz particles for 20min, spread plated in triplicate on King's B agar (KBA) medium and incubated at 30±2⁰C for 48 h. Bacterial cultures were maintained on the respective slants. After incubation at 28–30⁰C for 2–3 days, bacterial colonies were counted. Representative colonies were selected on the basis of distinct morphological characteristics, including pigments, colony form, elevation and margin; texture and opacity. A predominant yellowish white colony that

fluoresced under UV light was purified and maintained on nutrient agar slants at 4⁰C. All the subsequent experiments were carried out after raising fresh cultures.

Identification of antibiotic producing soil microbes

Isolated bacterial strain was identified morphologically (shape, Gram staining, spore staining, spore shape, sporangium dilatation and motility) and biochemically (sugar utilization, starch utilization, casein hydrolysis, in dole production, citrate utilization, methyl red, Voges-Proskauer (MR-VP), oxidase production, catalase production, nitrate reduction, gas production from glucose) according to the Bergey's Manual of Determinative Bacteriology.

Bioactive compounds production and Purification

Shake flask fermentation method was used for bacterial antibiotic production. Seventy-two hours old inoculum was prepared in Tryptic Soy Broth (pH 7.3) at concentration of 10% (v/v). Inoculum was added to the production medium and incubated for 24 hours in orbital shaker at 120 rpm.

After incubation, culture was centrifuged to get cell free supernatant and was further used for antimicrobial activity.

Antimicrobial activity by agar diffusion assay

Agar well diffusion method was used to check the cultures for the production of antimicrobial metabolites. Twenty-four hours fresh cultures of test pathogens were diluted with pre-sterilized normal saline. A sterilized cotton swab was dipped in the diluted cultures and lawns were prepared over the agar surface. Wells were made in the inoculated plates

using sterile cork borer. About 80 µL cell free supernatants were added in the wells and the plates were incubated at 37⁰C for 24hours. After 24hours, the zones of inhibition were observed. The diameter of the zone of inhibition was measured in mm with well size of 6mm.

Results and Discussion

Isolation and screening of bacteria

After incubation growth was observed on all plates of nutrient agar. However only the greenish colored colonies, which are peculiar characteristics of *Pseudomonas* spp. were selected as potential isolates. Total 16 isolates were obtained from selected soil samples. These isolates were labeled as S1 to S16.

Identification of the isolate

After growth on nutrient agar isolate S3, S6, and S15 showed greenish yellow colored colonies by producing a diffusible pigment. The biochemical characters were performed by using standard methods described in Bergey's manual of determinative bacteriology. According to King, *et al.*, (1954) *Pseudomonas aeruginosa* colonies appear green to bluish-green due to production of pyocyanin pigments. The results obtained with morphological and biochemical characteristics (Table: 1) for S3, S6, and S15 were compared with the characters of reference *Pseudomonas aeruginosa* (Bergey's Manual of Determinative Bacteriology) and it was found that S15 exhibits more similarity with the *Pseudomonas aeruginosa*.

The result revealed that the soil isolates have ability to produce secondary metabolites (SM) which have bioactivity against bacterial pathogens. The bacterial isolates isolated from Marathwada region of Maharashtra have ability to produce secondary metabolites

against *B. subtilis*, *E. coli*, *S. pyogenes*, *S. aureus*, *P. vulgaris* and *K. pneumonia* shown in table no. 2 and figure No.1.

A three bacterial isolates efficient in bioactivity against selected human pathogen

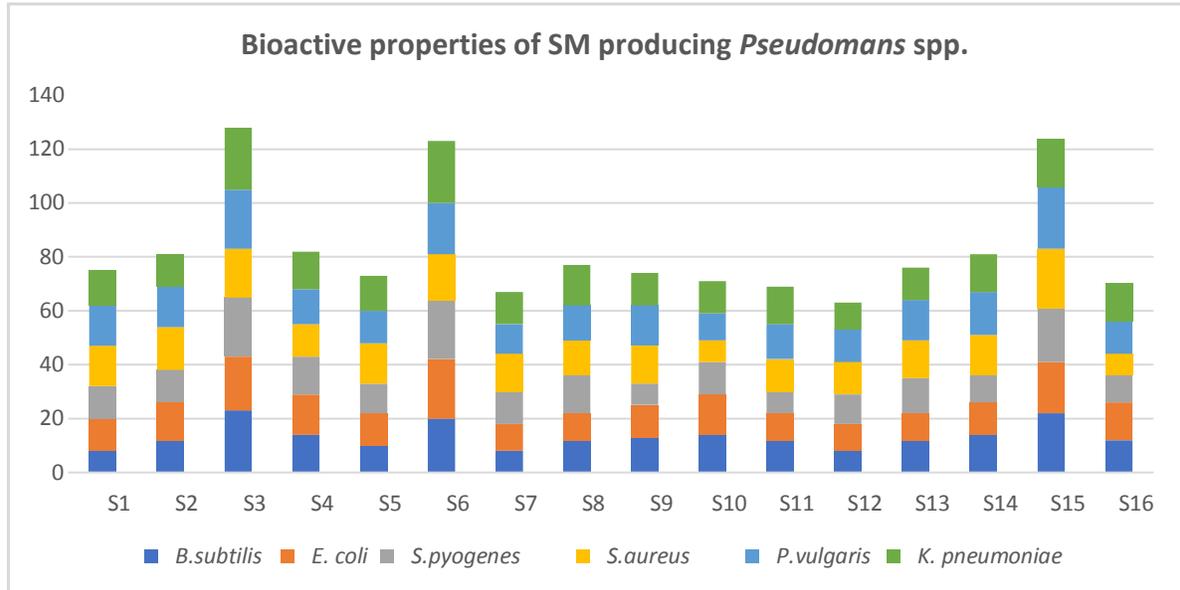
were identified as *Pseudomonas* spp by using criteria given in Bergey's Manuale of Systematic Bacteriology for identification. The identified *Pseudomonas* spp showed citrate, VP, gelatinase, catalase and oxidase test positive.

Table.1 Morphological and biochemical characterization of isolate

Sr. No	Morphological Character	Results	Biochemical test	Results
1.	Gram staining	Gram negative	Catalase test	+
2.	Motility	Motile	Oxidase test	+
3.	Cell shape	Rod	Sugar utilization test	-
4.	Greenish pigment	Present	Citrate utilization test	+
5.	Capsule	Absent	Casein hydrolysis test	-
6.	Spore	Absent		

Table.2 Bioactive properties of secondary metabolites (SM) producing Bacterial isolates (Zone of inhibition in mm)

Isolates	<i>B.subtilis</i>	<i>E.coli</i>	<i>S.pyrogenes</i>	<i>S. aureus</i>	<i>P.vulgaris</i>	<i>K. pneumoniae</i>
S1	8	12	12	15	15	13
S2	12	14	12	16	25	25
S3	23	17	22	18	22	23
S4	14	16	14	12	18	20
S5	10	12	11	15	12	13
S6	20	22	19	17	18	20
S7	8	10	12	14	11	12
S8	12	10	14	13	13	15
S9	13	12	8	14	15	12
S10	14	15	12	8	10	12
S11	12	10	8	12	13	14
S12	8	10	11	12	12	10
S13	12	10	13	14	15	12
S14	14	12	10	15	16	14
S15	17	19	20	22	20	18
S16	12	14	10	8	12	14

Fig.1 Antibacterial potential of *Pseudomonas* spp. from soil samples

The presence of such bioactive compound produced by *Pseudomonas* spp. might have novel structure, which may be responsible for its broad-spectrum microbial activity against human pathogen. The isolated and purified compound from *Pseudomonas* spp. strains in present investigation indicates that compound may have unique structure therefore it shows broad spectrum activity acting against tested human pathogen. The complete structure elucidation by advance instrumentation techniques is essential for explaining its uniqueness. Thus *Pseudomonas* spp. has moderate application in development of pharmacological lead compound against multiple antibiotic resistance human pathogens.

Antibiotics produced by *Pseudomonas* and *Bacillus* species play important role in biological control (McLoughlin, *et al.*, 1992; Buysens, *et al.*, 1996; Milner, *et al.*, 1996; Nielsen, *et al.*, 1988). Growth inhibition of the phytopathogen *Fusarium oxysporum* by *Bacillus subtilis* with production of the antibiotic 'bulbiformin' was reported by Vasudeva, (1952). Aska and Shoda (1996)

observed *Bacillus subtilis* RB14 as effective antagonist against *Rhizoctonia solani* with production of antibiotics 'iturinA' and 'surfactin'. The phenazine was dominant factor in disease suppression by *Pseudomonas fluorescens* 2-79 and M4-80R (Hamdan, *et al.*, 1991). Kumari and Srivastava (1999) concluded that, production of antimicrobial compound 2,4- diacetyl phloroglucinol (DAPG) and phenazine-1-carboxylic acid (PCA) by *Pseudomonas fluorescens* were responsible for control of black root rot of tobacco. Mazzola, *et al.*, (1995) observed same fact in case of biocontrol of take-all of wheat. Nielsen, *et al.*, (1998) observed production of a lipopeptide antibiotic-viscosinamide and 2,4 diacetylphloroglucinol as the apparent mechanisms of biocontrol of pre-emergence damping-off caused by *Pythium litinum* and *Rhizoctonia solani*, by *Pseudomonas fluorescens* isolated from sugar beet rhizosphere. Samanta and Dutta (2004) observed that, the crude extract of *Pseudomonas* spp. Mpf-1 inhibited the growth of *Sclerotinia sclerotiorum* by 84% and reduced the incidence of Sclerotinia stem rot of mustard. *Bacillus subtilis* inhibited the

growth of many fungal phytopathogens by antibiotic production (Podile, *et al.*, 1988).

Bioactive secondary metabolites production is one of the most important mechanisms of antimicrobial activity and biological control of microbial pathogens by *Pseudomonas* spp. The antibacterial activities achieved in this study indicate that the isolated strains have potential to produce diverse array of antibacterial compounds that can be useful for many great applications and must be explored extensively.

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