



## Isolation, characterization, and functional groups analysis of *Pseudoxanthomonas indica* RSA-23 from rhizosphere soil

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### ABSTRACT

In the present study, *Pseudoxanthomonas* species were isolated from rhizosphere soil samples of two selected medicinal plants, such as *Alternanthera sessilis* Linn. and *Leucas aspera* (Wild) Linn., which were collected from agricultural fields at Kundagol, Dharwad, Karnataka. *Pseudoxanthomonas* sp. is Gram negative, aerobic, motile, non-spore forming rod shaped bacterium and it was designated as *Pseudoxanthomonas* strain RSA-23; it was found to possess broad spectrum of antimicrobial activity against pathogenic microorganisms. Furthermore, the most potent strain RSA-23 was characterized by physiological, biochemical, and 16S ribosomal RNA gene sequencing. The 16S ribosomal RNA gene sequencing and analysis of phylogenetic tree showed 100% sequence similarity with *Pseudoxanthomonas indica* (KT204489). The methanol extract of *Pseudoxanthomonas* strain RSA-23 was analyzed through UV-spectroscopy and Fourier transform infrared spectroscopy (FTIR). The UV-Vis. spectra revealed the presence of indole and the presence of different functional groups, such as aldehydes, amines, and alkyl halides, were indicated by FTIR spectra.

### INTRODUCTION

Rhizosphere is the portion of soil which surrounds a living root. The rhizosphere soil is different from the soil away from the root zone in terms of chemical and physical nature and is always influenced by organic and inorganic exudates from the roots which affect the inhabited microbial communities surrounding it (Kent and Triplett, 2002). The rhizosphere contains both beneficial and harmful pathogenic microorganisms. The microorganisms residing in rhizosphere area are mycorrhizal fungi, nitrogen fixing bacteria, plant growth promoting rhizobacteria (PGPR), protozoa, actinomycetes, and harmful microorganisms which includes oomycetes, pathogenic bacteria, nematodes, and other human pathogenic microbes (Kaestli *et al.*, 2011; Teplitski *et al.*, 2012).

There are obvious differences between the rhizosphere and non-rhizosphere soils of the plants. The microbial association of rhizosphere soil is always higher than non-rhizosphere soil. The rhizosphere soil of medicinal plants has a structurally and functionally distinct microbial community and this distinct microbiota is due to the secretion of unique bioactive secondary metabolites. These unique secondary metabolites may be responsible for diverse microbial association. The plant *Leucas aspera* (Wild) Linn. is a member of the family *Lamiaceae*. The plant is an annual herb with quadrangular stem, the leaves are sessile, and flowers are white in color (Hooker, 1984). The plant has antimicrobial, antifungal, cytotoxic, and antioxidant properties (Mangathayaru, 2005). The plant *Leucas aspera* (Wild) Linn. was traditionally being used as antipyretic and insecticides, the leaves were used in chronic skin eruptions and snake bite (Rai *et al.*, 2005). *Alternanthera sessilis* Linn. is a leafy vegetable, generally found near tropical and sub-tropical areas. The plant is herbaceous with annual or perennial nature. The plant has antibacterial, cytotoxic, and anti-allergic properties (Ullah *et al.*, 2013).

The study of bacteria inhabitant of rhizosphere soil is important because of their effect on plant growth, production of

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industrially and medicinally useful metabolites (Rayees *et al.*, 2013). The yield and quantity of compounds of medicinal plants, nutrient uptake, and disease resistance are largely influenced by rhizosphere microorganisms (Bafana and Lohiya, 2013). In plant rhizosphere region, aerobic, gram positive, and sporulating bacteria are less as compared to anaerobic, Gram negative, and non-sporulating bacteria (Zakaria and Anawar, 2014) having a great symbiotic association to plants. These microorganisms take the exudates of plants as food, plants also takes some organic elements from the microorganisms for avoiding their nutrient deficiency for better growth and development.

The *Pseudoxanthomonas* is Gram negative, aerobic, atrichous, rod shaped, and non-spore forming bacteria which was first reported by Finkmann from experimental biofilters (Merckx *et al.*, 1987). Taxonomically, the genus *Pseudoxanthomonas* belongs to the phylum *Proteobacteria* class *Gammaproteobacteria* and family *Xanthomonadaceae* (Finkmann *et al.*, 2000). This genus is phylogenetically related to the genera *Xanthomonas*, *Xylella*, and *Stenotrophomonas*. Members of this genus have iso  $C_{15:0}$  and anteiso  $C_{15:0}$  as major fatty acids and ubiquinone (Q8) as major respiratory quinone (Soo *et al.*, 2015). Ecologically, the genus *Pseudoxanthomonas* is important due to the ability to degrade nitrate, nitrite, and different hydrocarbons, including ethyl-benzene, toluene, and benzene (Thierry *et al.*, 2004). In addition to this, *Pseudoxanthomonas* has various other applications in science in being used in the field of medical science, pharmaceutical science, food science, bioremediation, biodegradation and in agricultural field like PGPR for growth and development of plants without any use of chemical fertilizers. The present study aims to isolate *Pseudoxanthomonas* sp. from two medicinal plants to elucidate their antimicrobial activity against various pathogenic bacteria and fungi and the molecular characterization of the potent isolate using 16S rRNA gene sequencing.

## MATERIALS AND METHODS

### Collection and processing of sample

Around 500 g of soil samples were collected in sterile polythene bags from rhizosphere of two medicinal plants, namely, *A. sessilis* Linn. and *L. aspera* (Wild) Linn. from agricultural fields of Gudgeri, Kundagol, Dharwad. The soil samples were then labeled and air-dried at room temperature for 3 to 4 days. The cleaned and air dried samples were refilled in the respective polythene bags and stored at 4°C for further studies (Fatima *et al.*, 2017).

### Isolation of microorganisms

Each soil sample was serially diluted and 100  $\mu$ l of aliquots from  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ , and  $10^{-6}$  were spread evenly on nutrient agar (NA); pH 7.0 and czapek dox agar media aseptically. Both the media were supplemented with antibiotics and antifungal, such as Amphotericin B-50 and Tetracyclin (25  $\mu$ g/ml each), to inhibit the growth of unwanted bacteria and fungi. The plates were incubated at 30°C for 3 days and observed periodically for the growth of microbial strains and maintained in nutrient agar slants at 4°C for subsequent studies (Fatima *et al.*, 2017).

### Primary screening for antimicrobial activities

During the primary screening, the isolates were screened against pathogenic bacterial strains by perpendicular streak (cross streak) method according to Saravanakumar *et al.* (2014). In this method, nutrient agar medium was used and each plate was streaked with individual isolates along the diameter of the plate and incubated at 30°C for 4 days. Later, 24 hour fresh sub-cultured fungi and bacteria, viz., *Aspergillus fumigatus* (MTCC8877), *Enterobacter aerogenes* (ATTC2822), *Staphylococcus epidermidis* (MTCC435), and *Shigella flexneri* (MTCC1457) were prepared and streaked perpendicular to the isolates and incubated at 37°C for 48 hours.

### Morphological, physiological, and biochemical characterization

Morphological and physiological characterization was done by checking the growth in the range of temperature from 20°C to 40°C and at different pH level from pH 6.0 to pH 8.0. The biochemical analysis of hydrolysis tests were conducted on R2A medium supplemented with starch (1% w/v), casein (5% w/v), urea, and gelatin. Oxidase and catalase activity were tested and tests for the utilization of carbon sources such as D-glucose, maltose, D-mannose, L-arabinose, D-galactose, and citrate were done according to Yoo *et al.* (2007).

### DNA extraction and 16S rRNA gene sequencing

The genomic DNA was extracted from the fresh culture of the potent isolate. The 16S rRNA genes were amplified using primers. The forward primer 5'-AGAGTTTGATCMTGGCTCAG-3' (400 ng) and 16S reverse primer 5'-TACGGYTACCTTGTTACGACTT-3' (400 ng). The following condition were used for the PCR amplification; initial denaturation of 25 cycles at 96°C for 5 minutes, denaturation at 96°C for 1 minutes, hybridization at 50°C for 1 minutes, and elongation (final extension) 60°C for 2 minutes as described by Yoon *et al.* (1998). The PCR products were electrophoresed on 1% agarose gel with 500 bp DNA ladder as the size reference. Purified PCR amplicons were sequenced (sequence machine, Applied Biosystems, Sanger Sequencing 3500 Series, Genetic Analyzer) and used to interrogate the National Centre for Biotechnology Information (NCBI) database via BLASTN web portal according to Saravanakumar *et al.* (2014). The DNA sequences were aligned and a phylogenetic tree was constructed by the neighbor joining method using the software MEGA 7.0 by the method of Kumar *et al.* (2016).

### Fermentation and extraction of bioactive compound

The isolated strain was grown in nutrient broth (pH 7.0) and incubated at 30°C in a rotary shaker. After fermentation, the biomass was centrifuged at 5,000 rpm for 20 minutes and washed three times with sterile distilled water. The intra-cellular bioactive compounds were then extracted through solid-liquid extraction method. The biomass was suspended in a little amount of methanol and ground with a pestle and mortar. Methanol was added to the ground cells (1:1 w/v) and the mixture was shaken vigorously overnight. The extract was then filtered through a blotting paper and the filtrate was evaporated using a rotary evaporator at 50°C. The concentrated extract was then transferred into glass screw cap tubes and storage was done at 4°C for further

use (Saravanakumar *et al.*, 2014). Later, the crude extract was subjected to silica gel column chromatography and eluted with mixtures of CHCl<sub>3</sub>:CH<sub>3</sub>OH (Chloroform:Methanol).

### Ultraviolet visible (UV-Vis) spectroscopy

The qualitative analysis of the crude methanol extract was performed by UV-visible spectroscopy for the probable determination of natural compounds using a Jasco V-670 UV/VIS spectrophotometer.

### Fourier transform infrared resonance (FT-IR) spectral analysis

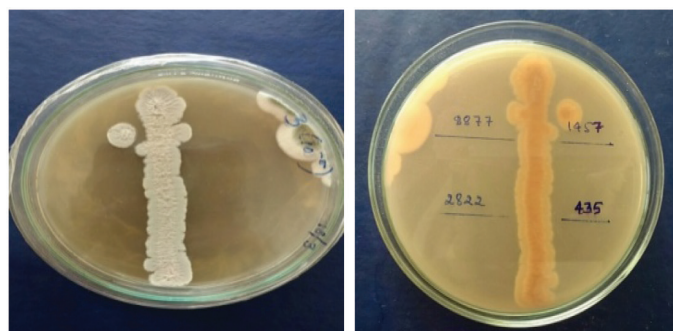
The biological functional groups present in the methanol extract was interpreted by FT-IR spectroscopy, using KBr; mixed with extract and compressed to make thin discs to be analyzed in prominent infrared resonance spectra in the range of 400 to 4,000 cm<sup>-1</sup>. The Fourier transform infrared (FTIR) spectrum of active extract was detected using Nicolet 6700 (Thermo scientific Class 1 Laser product-APW 1300066) in University Scientific and Instruments Centre (USIC), Karnatak University, Dharwad.

## RESULTS AND DISCUSSION

Totally, 47 microorganisms were isolated from rhizosphere soils on NA. Among them, only the colonies showing yellow color with circular and entire margins were sub-cultured. Different studies showed the isolation of various species of *Pseudoxanthomonas* from different samples such as bio-filters (Finkmann *et al.*, 2000), sludge, soil (Thierry *et al.*, 2004), and compost (Weon *et al.*, 2006). Among all the isolates, only 25 isolates were found to be Gram-negative strains and the strain RSA-23 showed 100% inhibition for all the pathogenic organisms during primary screening (Fig. 1), this was used for further analysis. Rest all organisms showed moderate inhibition against the pathogenic organisms (Table 1).

### Morphological, physiological, and biochemical characterization

The detailed morphological, physiological, and biochemical properties of the strain RSA-23 was shown in the Table 2. The pH which favored optimum growth of the strain RSA-23 was found at pH 7.0. But, there was no positive effect on



**Figure 1.** Primary screening of strain RSA-23 using cross streak method against *A. fumigatus* (MTCC8877), *S. flexneri* (MTCC1457), *E. aerogenes* (ATTC2822), and *S. epidermidis* (MTCC435).

growth at the pH below and above 7.0. The effect of temperature on the strain RSA-23 showed a relatively good growth at 30°C. Our results were confirmative with the result of Jianli *et al.* (2014), where *Pseudoxanthomonas wuyuanensis* showed optimum growth at 30°C to 37°C at pH 6.0 to 7.0. The strain RSA-23 showed the positive test for starch and aesculin, whereas casein, gelatin, and urea were negative. The carbon sources most utilized by the strain RSA-23 were D-glucose, maltose, and D-mannose. There was no growth observed in L-arabinose and D-galactose. The organism was found positive for oxidase and catalase. A similar result was found by Soo *et al.* (2015) for *Pseudoxanthomonas sangjuensis* isolated from greenhouse soil.

### 16S ribosomal RNA gene sequencing

The genomic DNA was isolated from the strain RSA-23 and analyzed by 16S rRNA gene sequencing with the help of PCR amplification. 16S rRNA is a small segment of RNA which is associated with 30S small subunit of bacterial ribosome and has a wide use in identification of microorganisms up to species level. The complete sequence obtained was 1,383 base pair in length. The sequence was submitted to NCBI with the accession number MK905527, which was later, subjected to search of close relatives through Nucleotide BLAST (BLASTN) in NCBI. The analysis of

**Table 1.** Activity of isolated strains against different test pathogens.

Strain No.	<i>A. fumigatus</i> (MTCC8877)	<i>E. aerogenes</i> (ATTC2822)	<i>S. epidermidis</i> (MTCC435)	<i>S. flexneri</i> (MTCC1457)
RSA-1	-	++	++	-
RSA-2	-	-	-	+
RSA-3	-	-	-	-
RSA-4	-	-	+	+
RSA-5	+	-	-	-
RSA-6	-	-	-	-
RSA-7	+	-	-	+
RSA-8	+	-	++	-
RSA-9	-	++	+	-
RSA-10	+	-	-	-
RSA-11	-	-	-	-
RSA-12	+	-	++	-
RSA-13	-	-	-	-
RSA-14	-	+	-	-
RSA-15	-	-	-	-
RSA-16	+	+	++	-
RSA-17	-	+	-	+
RSA-18	-	-	-	-
RSA-19	+	-	++	-
RSA-20	-	-	-	-
RSA-21	++	-	-	+
RSA-22	-	-	-	++
RSA-23	++	++	++	++
RSA-24	-	-	-	+
RSA-25	++	-	-	-

(++ Good antimicrobial activity; + Moderate antimicrobial activity; - No antimicrobial activity).

**Table 2.** Morphological, physiological and biochemical characterization of strain RSA-23.

Characteristics of strain RSA-23	
Habitat	Soil
Colony appearance	Yellow and circular colonies with entire margin
Motility	Motile
Gram staining	Gram negative
pH	Growth of RSA-23
pH 6.0	-
pH 6.5	+
pH 7.0	++
pH 7.5	W
pH 8.0	-
Growth at different temperature (°C)	
Temperature	Growth of RSA-23
20°C	-
25°C	W
30°C	++
35°C	W
40°C	-
Biochemical tests for strain RSA-23	
Starch	+
Casein	-
Urea	-
Gelatin	-
Aesculin	+
Assimilation of carbohydrates	
D-Glucose	+
Maltose	+
D-Mannose	+
L-Arabinose	-
D-Galactose	-
Citrate	-
Oxidase	+
Catalase	+

(- No growth; W = Weak growth; +: Normal growth; ++: Optimum growth).

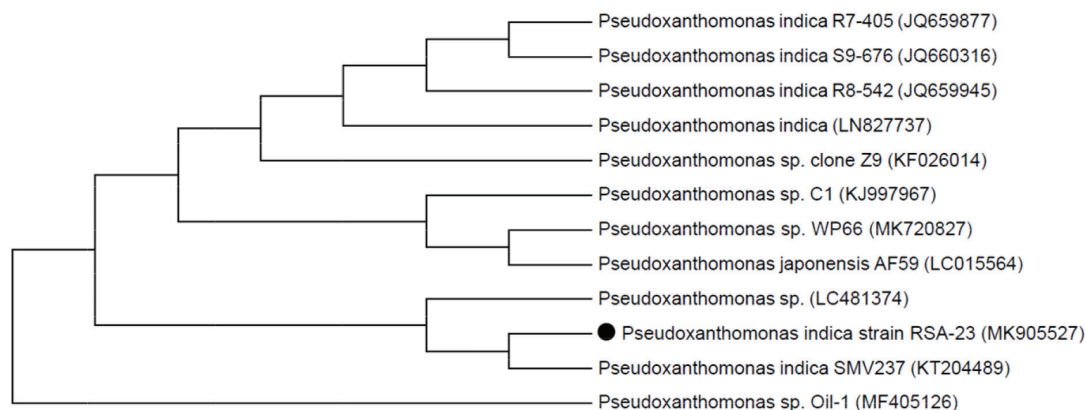
16S rRNA gene sequence of the strain RSA-23 revealed 100% sequence similarity with *Pseudoxanthomonas indica* strain SMV237 (KT204489) and 99.35% sequence similarity with *Pseudoxanthomonas* sp. (LC481374), *Pseudoxanthomonas* sp. strain WP66 (MK720827), *Pseudoxanthomonas japonensis* strain AF59 (LC015564). Further a phylogenetic tree was constructed through neighbor joining method with obtained gene sequences, the analysis of phylogenetic tree revealed the strain RSA-23 fell within the cluster of the genus *Pseudoxanthomonas* and formed a branch with *P. indica* SMV237 (KT204489) (Fig. 2). This result can be correlated with the results of Mohapatra *et al.* (2018), where the strain KAs 5-3<sup>T</sup> gave the highest sequence similarity (99.25%) with the members of the genus *Pseudoxanthomonas mexicana* (AMX 26B<sup>T</sup>).

### UV absorption analysis of RSA-23 methanol extract

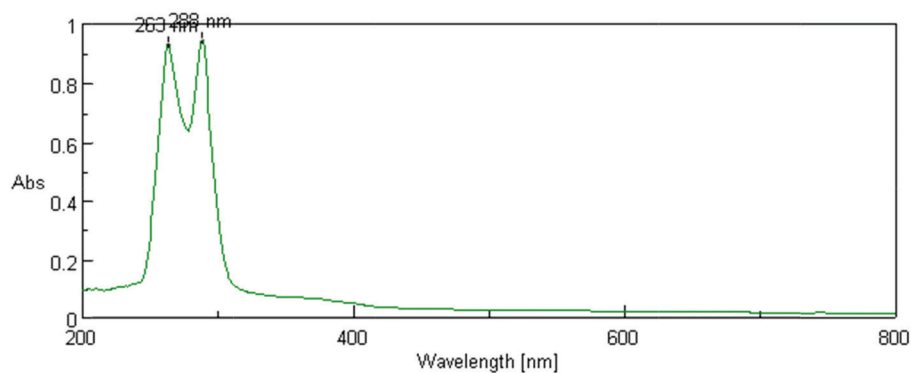
The UV-visible spectroscopy was used for quantitative analysis. Generally, the aromatic compounds are powerful chromophores in the UV range; the natural compounds can also be determined by using UV-visible spectroscopy (Kemp, 1991). The methanol extract of the *P. indica* RSA-23 exhibited maximum UV absorption at 263 nm and 288 nm as shown in Figure 3. The peaks at 263nm and 288nm can be attributed to an aromatic heterocyclic compound indole (Kemp, 1991a; 1991b). Indole can be produced by a variety of bacteria and works as an intercellular signal molecule in performing various aspects of bacterial physiology, including plasmid stability and virulence (Lee and Lee, 2010).

### FTIR analysis of RSA-23 methanol extract

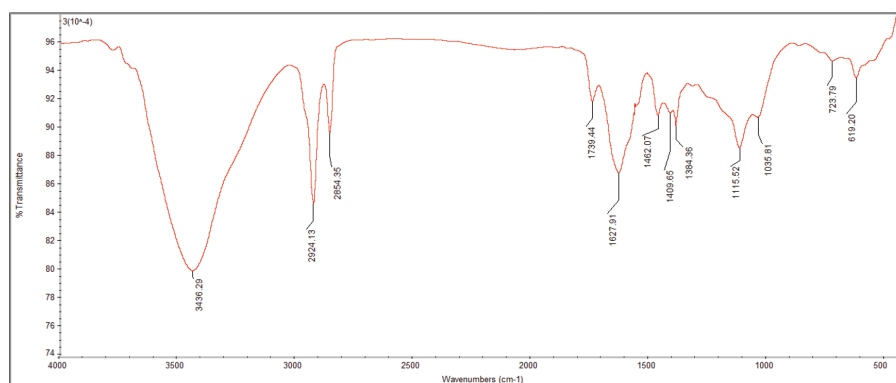
The FTIR spectrum of *P. indica* RSA-23 methanol extract used to determine the functional groups by active components, based on the peak values in the region of infrared radiation as shown in the Figure 4 and Table 3. It revealed the presence of different functional groups based on the absorption peaks at 619 and 723 cm<sup>-1</sup> due to C-Br stretching alkyl groups and C-Cl stretch alkyl halides, respectively, 1,035 cm<sup>-1</sup> C-N stretch alkyl halides, 1,115 cm<sup>-1</sup> C-O stretch carboxylic acids/esters, ethers, 1,384 cm<sup>-1</sup> C-H rock alkanes/CH<sub>2</sub> wag modes, 1,409 cm<sup>-1</sup> C-C stretch aromatics, 1,462 cm<sup>-1</sup> C-H bend alkanes, 1,627 cm<sup>-1</sup> N-H bend 1<sup>o</sup> amine, 1,739 cm<sup>-1</sup> C=O stretch esters or saturated aliphatics, 2,854 cm<sup>-1</sup> C-H stretch alkanes, 2,924 cm<sup>-1</sup> H-C=O: C-H stretch



**Figure 2.** Dendrogram indicating the phylogenetic relation of the *Pseudoxanthomonas indica* RSA-23.



**Figure 3.** UV-Spectrum of methanol extract of *Pseudoxanthomonas indica*. RSA-23.



**Figure 4.** FTIR spectrum of methanol extract of *Pseudoxanthomonas indica*. RSA-23.

**Table 3.** FTIR absorption peaks and their associated functional groups.

Sl. no.	Absorption peak (cm <sup>-1</sup> )	Functional groups
1	619	C-Br stretching alkyl groups
2	723	C-Cl stretch alkyl halides
3	1,035	C-N stretch alkyl halides
4	1,115	C-O stretch carboxylic acids/esters ethers
5	1,384	C-H rock alkanes/ CH <sub>2</sub> wag modes
6	1,409	C-C stretch aromatics
7	1,462	C-H bend alkanes
8	1,627	N-H bend 1 <sup>o</sup> amine
9	1,739	C=O stretch esters, saturated aliphatics
10	2,854	C-H stretch alkanes
11	2,924	H-C=O: C-H stretch aldehydes
12	3,436	O-H stretch alcohols

aldehydes, and 3,436 cm<sup>-1</sup> O-H stretch alcohols. This result was compared with the similar results of *Fatima et al.* (2017).

## CONCLUSION

The current study shows that the isolated strain *P. indica* RSA-23 from rhizosphere soil samples of agricultural field of Kundagol region has the potential to act against pathogenic microorganisms. Here, we found that agricultural field of Gudgeri, Kundagol, Dharwad (District) is a good region of biodiversity and has been adequately acceptable due to its vast floral and

microbial diversity. Results show that the strain *P. indica* RSA-23 is dominant against all the active test pathogens. Thus, the study on this isolate can further be explored for the development of new antibiotic drugs to treat infectious diseases caused by pathogenic and drug resistant strains and can also be analyzed if this strain has any PGPR or weed biocontrol activity.

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## CONFLICTS OF INTEREST

Authors declare that there are no conflicts of interest.

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