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Production and characterization of antimicrobials from isolate *Pantoea agglomerans* of *Medicago sativa* plant rhizosphere soil

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Abstract
Due to rise in drug resistance among pathogens, there is always an urge to look for new drug alternatives. So in this study we aimed to identify the unexplored rhizosphere microflora of alfalfa plant for new antimicrobials. With initial screening for isolates from rhizosphere region for antibacterial activity against selected bacterial pathogens, the isolate AL10 had better activity selected for this study. The isolate mass was cultured and secondary metabolites were extracted using ethyl acetate and subjected to FTIR and GC-MS analysis. Based on functional diversity analysis, the isolate subjected to anti-bacterial activity revealed significant activity against *Streptococcus pneumonia, Klebsiella, S. aureus* with zone of inhibition in the range of18-20 mm. Based on GC-MS analysis report ten compounds were identified and 1-Octadecane and 1-nonadecanol were found to be responsible for bio-activity. FT-IR results showed that N-H stretching functional group was dominantly present in the extract. Molecular identification of the isolate by 16S rRNA sequencing showed the isolate as *Pantoea agglomerans.* The results showed that the isolate *P.agglomerans,* gram negative bacteria had wide antibacterial activity due to 1-Octadecane and 1-nonadecanol. Though Alfalfa plant has been described for various biological activities, this is a first report on rhizosphere region of plant reporting for antibacterial potential microbes.

Keywords: Alfalfa plant, Antibacterial activity, Gas chromatography-mass spectrometry, *Pantoea agglomerans,* Rhizosphere.

INTRODUCTION

Alfalfa means “father of all foods”, is a perennial herbaceous leguminous flowering plant. *Medicago sativa,* “Queen of Forage plant” can live upto 8 years, fixes high nitrogen than other plants due to its symbiotic association with microbes. The plant possesses deep root system which enables high water holding ability of plants and thereby preventing soil erosion. They are cultivated worldwide, used as fodder for cows for high protein content and fiber (Frame, 2005). *M. sativa*, a high-remedial value plant has been reported to contain bioactive compounds namely saponins, flavonoids, phytoestrogens, coumarins, alkaloids, amino acids, phytosterols, vitamins, digestive enzymes and terpenes (El-Khrisy *et al*., 1994; Bialy *et al*., 1999). These compounds might be responsible for various phytopharmacological activities and also in treatment of various diseases (Zhang *et al*., 2006; Bora and Anupam, 2011; Krakowska *et al*., 2017).

Several species of *Pantoea* have been both beneficial for plant growth and also harmful affecting plants causing bacterial blight, leaf lesions, and

dieback diseases (Walterson and Stavrinides, 2015). *Pantoea agglomerans* are soil dwelling, yellow pigmented (produces carotenoids), plant associated gram negative rod shaped bacteria (Mohammadi *et al*., 2012).*P.agglomerans* has been reported for its presence in soil, water, dust, dairy products, fish, insects, humans and animals (Suen *et al*., 2010; Prakash *et al*., 2015; Buyukcama *et al*., 2017). Malboobi *et al*., (2009) isolated *P.agglomerans* form the rhizosphere region of potato and evaluation studies revealed that phosphate solubilizing activity lead to higher biomass of potato when applied in combination with other isolates.

Researchers have shown that *P.agglomerans* has protective ability to prevent plant against *Pseudomonas syringaepv. Syringae* (Braun-Kiewnick *et al*., 2000), also plant growth promoting activities of various crops (Verma *et al*., 2001; Feng *et al*., 2006). The reason behind growth promotion in plants can be attributed due to alteration in root architecture thereby increases uptake of water, nutrients and also IAA production (Sergeeva *et al*., 2007; Kulkarni *et al*., 2013). The microbe also brings up soil aggregation and moisture control due to production of exopolysaccharides (Amellal *et al*., 1998). Reports are available proving the efficiency of *P. agglomerans* strains for phosphate solubilisation, therby increasing plant growth and yield of crops (Viruel *et al*., 2011; Khalimi *et al*., 2012; Silini-Cherif *et al*., 2012).

Initially functional diversity studies has been done (unpublished results) on the isolation of microbes from the rhizosphere of alfalfa plant. Out of 32 isolates, this isolate was selected for its amylase, cellulase, protease and phosphate solubilization activities.

Bacillus horikoshii was isolated and molecularly identified from rhizosphere region of Alfalfa plant possessed antibacterial compounds detected by GC-MS (Nisha *et al*., 2019). This study was aimed at culturing, extraction and identification of bioactive compounds of functionally diverse organism from rhizosphere soil region of Alfalfa plant (*Medicago sativa*) through GC-MS and FTIR analysis and its antibacterial activity.

MATERIALS AND METHODS

Sample collection: Soil samples were collected from the rhizosphere region of Alfalfa plant fields during June 2016 from Sulur, Coimbatore, Tamilnadu, India. Studieswere carried out from June 2016 to March 2017.

Isolation and identification of microorganism: One gram of the collected five soil samples were used for serial dilution to isolate microbes by spread plate method. To the nutrient agar plates, 0.1ml of serially diluted samples (10^{-1} to 10^{-7}) were plated, incubated at 37˚C for 24-72 hours. After incubation, bacterial isolates were checked for purity and preserved in glycerol stocks and as nutrient agar slants for further tests.

These colonies were observed for Gram's nature and morphological characters such as size, shape, color, texture, opacity, elevation, margin and mobility. They were further identified using biochemical methods as stated in Bergey's manual for characterization which includes Indole, Methyl Red, Voges Prauskaeur, citrate, urease, TSI slants etc.

Antibacterial activity: The antimicrobial activities of crude extracts of all isolated bacteria were tested against bacterial pathogens (*P.aeruginosa, Klebsiella sp, S. aureus, Proteus vulgaricus, S. pneumonia, E. coli, B. cereus*) by agar well diffusion method. Muller-Hinton agar (MHA) plates were prepared and the wells were made with sterile cork borer on the agar plates. The overnight grown nutrient broth cultures of all bacterial pathogens were uniformly swabbed on to the surface of MHA plates using sterile cotton swabs. Each 50 µl of cell free supernatants were aseptically incorporated into the well and the plates were incubated in an upright position at 37 °C for 24 h. After incubation, the plates were observed for zone of inhibition.

Production and extraction of the bioactive compounds: For obtaining the large biomass, the active strain of *P. agglomerans* were inoculated into 1 litre of nutrient agar medium and incubated in shaker at 30°C at 160 rpm for 36 h. After incubation, the media contents were centrifuged at 10,000 rpm for 10 minutes to obtain the cell free supernatant.

The cell free supernatant extracted with organic solvent – Ethyl acetate and extraction carried out with 3 volume of solvent for 2 h by using rotary shaker supernatant fractions were flash evaporated at 45°C temperature to ensure complete removal of solvent and the extracts were evaporated to dryness. The resulting residues were dissolved in small amount of respective solvents and stored at –20°C until further purified.

Molecular identification and phylogenetic analysis of the bioactive compound

Genomic DNA isolation: DNA isolation from bacterial isolate (*P. agglomerans*) was performed according to the cold spring harbour lab protocol. Briefly, the isolates were grown in Nutrient Broth (Himedia, India) for 24 h days at 37 °C. The contents were centrifuged and pellets washed with 1 ml Tris-EDTA (TE) buffer, resuspended in 500 µl TE buffer containing 1 mg/ml lysozyme, incubated for 2 hrs at 37 °C. About 75 µl of 10 % Sodium Dodecyl Sulfate (SDS) and 125 µl of 5 M NaCl were added to above mixture, centrifuged (10,000 rpm for 10 min at 37 °C) and incubated in ice cold ethanol (–70 °C) for 3 min, later in a 65 °C water bath for 3 min and on ice for 10 min. About 200 µg/ml of RNase added, incubated at 37 °C for 15

min and further 50 µg/ml of Proteinase K added and incubated at 37 °C for 30 min. Following centrifugation at 10,000 rpm for 10 min at 37°C, equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) added to upper aqueous phase. The upper phase was recovered and mixed with equal volume of chloroform/isoamyl alcohol (24:1). To the aqueous phase, 50 µl of 3 M sodium acetate, 300 µl of ice-cold isopropyl alcohol was added, incubated at -20 °C for 20 min. The DNA precipitated was pelleted, washed twice with 70 % ethanol, blot dried, resuspended in 40 µl of TE buffer and stored at -20°C.

Amplification of 16S rRNA gene: 16S rRNA genes were amplified from the extracted genomic DNA using the 8 F and 1541 R universal eubacterial primers designed to target the conserved regions in the genomic DNA of the isolates and amplify approximately 1.4 kb length gene. The forward primer 5`-AGAGTTTGATCCTGGCTCAG-3` and reverse primer 5`-AAGGAGGTGATCCAGCCGCA -3` were used for amplification (Farris and Olson, 2007).

The PCR mix contained 5 µl of 25 X PCR buffer, 4 µl of 25 mM $MgCl_2$, 5 µl of 5 µM 518 Forward primer and 5 µl of 5 µM 800 Revers Primer, 5 µl of 1 mMdNTP's, 0.5 µl of *Taq*DNApolymerase (Thermo Scientific, India) and 2 µl of genomic DNA. The reaction volume adjusted and made up to a final volume of 50 µl with sterile double-distilled water and amplified in an automated thermal cycler (Vapo protect Pro S, Eppendorf). The PCR conditions were an initial denaturation stage at 95 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 60 s, extension at 72 °C for 60 s and a final extension step at 72 °C for 10 min. Negative controls with no DNA template were included in all PCR experiments.

16S rRNA gene sequencing: The Polymerase Chain Reaction (PCR) products purified using Montage PCR Clean up kit (Millipore) and sequenced by Big Dye terminator cycle sequencing kit (Applied BioSystems, USA) and resolved by Applied Biosystems model 3730XL automated DNA sequencing system (Xcelris Laboratories, India).

Phylogenetic analysis: The 16s rRNA sequence blast was done using NCBI blast similarity search tool and for multiple sequence alignment MUSCLE 3.7 program was employed. The resulting aligned sequences were cured using the program Gblocks 0.91b.This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise). PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.

FTIR analysis: The purified bacterial extract was subjected to FTIR spectroscopic analysis (Perkin Elmer Lambda), equipped with KBr beam splitter with DTGS (Deuteratedtriglycine sulfate) detector.

GC-MS analysis: The Thermo MS DSQ II with DB 35- MS Capillary standard non- polar column and helium as carrier gas (1 ml/min) was employed for GC-MS analysis. Sample volume of 1µl, injector temperature at 260˚C and oven temperature of 70˚C (6 min) was employed. Compounds were identified based on mass spectrums using. National Institute Standard and Technology (NIST) database. Through NIST library, name, molecular weight and structure of the components of the test materials were ascertained.

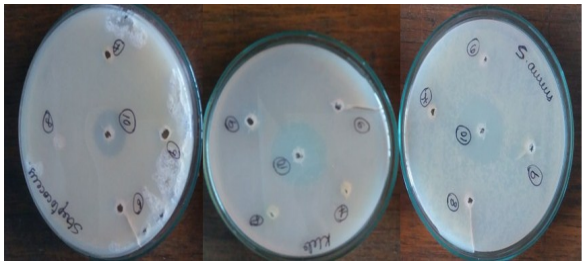

Fig. 1. *Showing antibacterial activity of AL10 against Staphylococccus pneumoniae, Klebsiella sp and Streptococcus aureus.*

RESULTS AND DISCUSSION

Antibacterial activity: The rhizosphere region of soil harbor a variety of microbial flora due to the nutrient compounds released from plants roots, thereby playing crucial role in soil function. The antibacterial activity of the crude extract of the isolate *P.agglomerans* studied against 7 clinical pathogens revealed significant zone of inhibition of 18, 20 and 19 mm against *Streptococcus pneumonia, Klebsiella sp, S. aureus* respectively (Fig. 1).

In concordance with our study results, similar results have been obtained for microbial isolate from various rhizosphere region of soil samples possessing significant anti-microbial activity. Jayapradha *et al*. (2009) reported the wide range of antibacterial activity of *Streptomycetes* sp. isolated from the rhizosphere soil of medicinal plants at

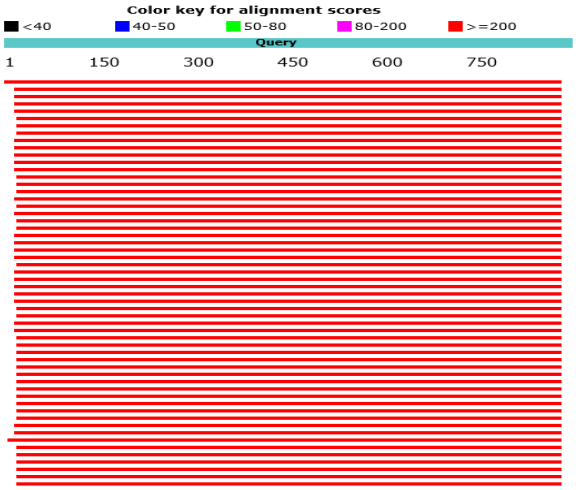

Fig. 2. *Showing multiple alignment scores of P. agglomerans.*

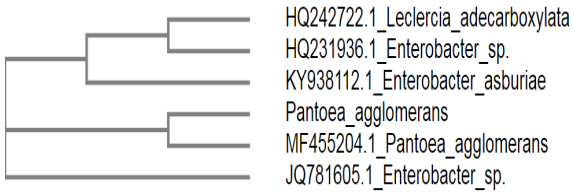

Fig. 3. *Phylogenetic tree of P. agglomerans based on the 16S rRNA gene sequencing.*

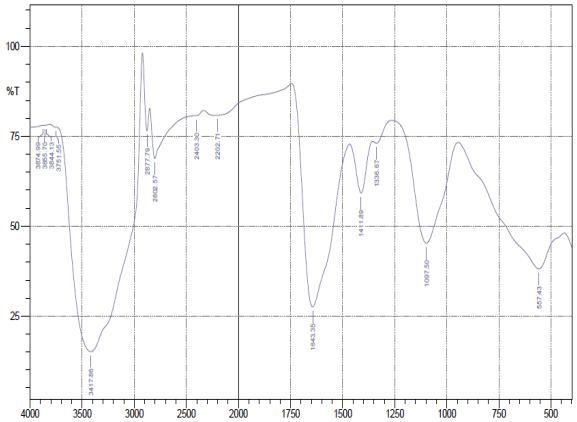

Fig. 4. *FTIR spectrum of P. agglomerans extract.*

Kolli hills of Tamil Nadu. Ryandini *et al*. (2018) isolated *Streptomyces* sp.from mangrove rhizosphere mud of Rhizophora mucronata from east Segara Anakan mud and reported significant activity on multipledrug resistant bacteria. Also Rajalakshmi and Mahesh, (2014) reported antimicrobial activity of *Aspergillus terrus* isolated from rhizosphere region of medicinal plants in and around Kuttralam, Tirunelveli. Upon GC-MS analysis, ten compounds were identified and tetracontane was

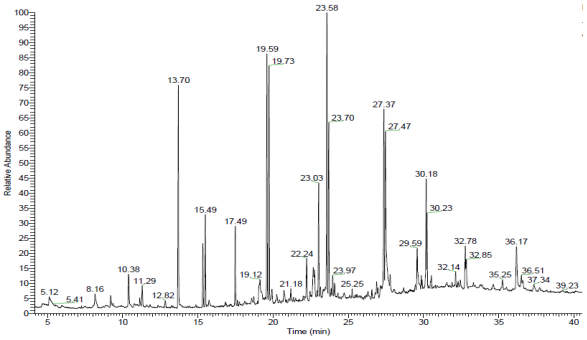

Fig. 5. *GCMS spectrum analysis of P. agglomerans extract.*

reported to be bioactive potential compound.

Molecular characterization of the isolates: The genomic DNA of the isolate isolated and subjected to 16S rRNA gene amplification for the species identification. PCR product of the length 1,400 bp purified and sequenced in Yaazh Xenomics lab, Coimbatore. The 16S rRNA sequences of the isolate subjected to BLAST analysis using mega blast tool of GenBank (http://www.ncbi.nlm.nih.gov/). Among different species comprising of closet neighbouring strains in NCBI-BLAST analysis used in the phylogenetic analysis. The phylogenetic trees were constructed based on the neighbour joining method and percentage differences in the genetic relationships between the neighbouring strains of the two samples were analyzed.

Results revealed that the 16S rRNA partial gene sequence of the isolate showed 100 % similarity with *P. agglomerans* (Fig. 2 and 3).

FTIR analysis: Fig 4 depicts the FTIR analysis of the *P. agglomerans* extract showing strong peaks

Table 1. *FTIR analysis of P. agglomerans extract.*

S.N.	Peak	Corr. Intensity	Corr.area	Functional group	Type of vibration
1	1658.78	16.466	15.184	C=C	stretch
2	2926.01	15.144	13.159	C-H	stretch
4	715.59	11.4652	21.998	=C-H	Bending
5	1550.77	7.518	4.53	C=C	Stretch
6	1126.43	4.671	2.412	C-O	stretch
7	1409.90	3.297	1.364	-C-H	bending
8	3441.01	3.379	9.616	O-H	Stretch, H- bonded
9	2860.43	2.75	1.018	C-H	stretch

Table 2. Showing GCMS analysis of compounds obtained from *P. agglomerans* extract.

S.N.	RT	Name of the compound	Molecular Formula	MW	Peak area (%)
1.	8.18	Dodecane	$C_{12}H_{26}$	170	0.99
2.	10.40	4-Cyano-2H-1-benzothiopyran	$C_{10}H_7NS$	173	1.77
3.	13.70	2-tert-Butyl-4-isopropyl-5-methylphenol	$C_{14}H_{22}O$	206	8.60
4.	15.49	Hexadecane	$C_{16}H_{34}$	226	5.22
5.	19.49	1-Octadecane	$C_{18}H_{36}$	252	15.15
6.	23.58	1-Nonadecanol	$C1_9H_{40}O$	284	15.32
7.	27.47	1-Heneicosyl formate	$C_{22}H_{44}O_2$	340	12.41
8.	30.18	1-Docosanol	$C_{22}H_{46}O$	326	6.11
9.	32.80	2- Hexadecanol	$C_{16}H_{34}O$	242	2.94
10.	36.20	9-Octadecenamide	$C_{18}H_{35}NO$	281	2.89

Table 3. Activity of compounds identified in *P. agglomerans* extract.

S.N.	RT	Name of the compound	Compound nature	Activity
1.	8.18	Dodecane	Alkane hydrocarbon	Solvent Antibacterial activity
2.	10.40	4-Cyano-2H-1-benzothiopyran	Bicyclic benzene	Antimalarial Anti-coagulant
3.	13.70	2-tert-Butyl-4-isopropyl-5-methylphenol	Phenol	Antioxidant
4.	15.49	Hexadecane	Alkane hydrocarbon	Detonation of diesel fuel
5.	19.49	1-Octadecane	alkane hydrocarbon	Antibacterial activity Antifungal activity
6.	23.58	1-Nonadecanol	Fatty alcohol	Antimicrobial and cytotoxic compound
7.	27.47	1-Heneicosyl formate	formic acid	Biocontrol activity
8.	30.18	1-Docosanol	Fatty alcohol	Anti-viral drug
9.	32.80	2- Hexadecanol	Fatty alcohol	Emulsifier
10.	36.20	9-Octadecenamide	Fatty acid	Treatment for mood and sleep

at 3417 cm^{-1}, 1643.35cm^{-1}, 1097.5 cm^{-1} and 1658.78cm^{-1}, 2926 cm^{-1}, 715cm^{-1} respectively. Major group was found to be N-H stretching at 3417 cm^{-1} (Table 1). The FTIR results elucidated an array of functional groups at a frequency ranges indicating the presence of functional groups corresponding to aromatic alkenes, aliphatic amines, compounds with aromatic rings, alkynes, amides, alcohols and phenols in partial fraction of *P. agglomerans* cell free supernatant.

GC-MS analysis: Fig 5 depicts the GC-MS analysis of *P. agglomerans* extract revealing presence of thirty two peaks and ten compounds were characterized and identified by comparison of the mass spectra of the constituents with the NIST library (Table 2 and 3). The retention times (RT) are represented in minutes.

The major constituent of *P. agglomerans* extract was found to be 1-octadecane, an alkane hydrocarbon at retention time 19.49 has highest intensity of 15.15. Studies have reported the antibacterial and anti-fungal activity of octadecane (Nazemi *et al*., 2010; Guo *et al*., 2008). The fatty alcohol 1-Nonadecanol was also found highest with intensity of 15.32 and retention time 23.58 minutes, has been reported for antimicrobial and cytotoxic properties (Dalli *et al*., 2007).

2-tert-Butyl-4-isopropyl-5-methylphenol, a phenolic compound possesses antioxidant activity has been recorded at retention time of 13.7 min with intensity of 8.6. The formic acid 1-Heneicosyl formate found with intensity of 12.41 and retention time of 27.47 minutes. Fatty alcohol such as 1-Docosanol has been found in extract of *P.agglomerans* with intensity of 6.11 and retention time of 30.80 minutes. 2- Hexadecanol has been recorded at retention time of 32.80 minutes. 9-Octadecenamide, a fatty acid was found at retention time of 36.20 minutes.

B. horikoshii identified from rhizosphere region of Alfalfa plant possessed antibacterial activity against *Klebsiella, Staphylococcus aureus* with zone of inhibition in the range of 17-18 mm and six compounds were detected by GC-MS and 11-Octadecanal responsible for bio-activity (Nisha *et al*., 2019).

Conclusion

Based on molecular identification the active isolate of rhizosphere region of Alfaalfa plant identified as *P. agglomerans* and extract identification showed presence of 10 volatile compounds namely Dodecane, 4-Cyano-2H-1-benzothiopyran, 2-tert-Butyl-4-isopropyl-5-methylphenol, Hexadecane, 1-Octadecane, 1-Nonadecanol, 1-Heneicosyl formate, 1-Docosanol, 2- Hexadecanol and 9-Octadecenamide. And study on the antimicrobial activity showed that activity could be due to presence of 1-Octadecane and 1-nonadecanol. Thus further *in vitro* and *in vivo* biological studies are required for anticancer medical applications in various fields. Through this study we were able to isolate new antimicrobials against potent pathogens.

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