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Bioactivity Assessment of potentially active Actinomycetes from Rhizospheric Soil

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Soil organisms have been serving as therapeutic potent components as they produce many bioactive natural products or secondary metabolites which act as antibiotics to fight against the evolving present day drug resistance along with their other important such as anti-microbial, anti-oxidant, anti-inflammatory, anti-androgenic and anticancer properties. In the present study a total of 65 actinomycetes were isolated from rhizospheric soil samples from four different locations of Rajasthan on Actinomycetes Isolation Agar (AIA) medium. Extraction of compounds using four solvents such as benzene, petroleum ether, chloroform and ethyl acetate were carried out, followed by partial purification of compounds using Thin Layer Chromatography (TLC). GC-MS technique uncovered the presence of many compounds having antimicrobial activity like antibacterial, antifungal, antioxidant, and many other activities. The potential of AIA26 isolate was tested for their antimicrobial activity in terms of inhibition zones (IZ) on 5 different indicator pathogens. It was observed that Benzene extract was active against *S. aureus* (IZ-24 mm) & *P. aeruginosa* (IZ-42 mm) and that of Chloroform extract was active against *P. aeruginosa* (IZ-18mm). No activity was recorded against *K. pneumonia*, *P. vulgaris*, and *B. subtilis*. The results of this study suggest that rhizospheric soil of Rajasthan is a potential source and reservoir of novel antibiotics.

Keywords: AIA26, Anti-microbial, GC-MS, Rajasthan, Soil organism

Introduction

Actinomycetes are saprophytic bacteria that are Gram positive organisms and are ubiquitous in soil as well as terrestrial habitats. They are appreciable contributors of complex biopolymers of organic matter, for example chitin and lignocellulose. They act as production house for numerous well known secondary metabolites, with many significant applications in the medical, agricultural and pharmaceutical industries, for example antibiotics, antitumor, anti-infection agents and other novel natural products. There are evidences of several reports that actinomycetes have been using as biological control for plants protecting them from pathogenic bacterial and fungal sp.[1,2] Secondary metabolites from microbes are broadly known for their biological activities for the well-being of humans. There are a plenty of secondary metabolites used as antibiotics, other medicinal purpose, toxins, pesticides, animal and plant growth modulators. Fleming's discovery of penicillin was the revolutionary invention in the golden era of antibiotic discovery.[3] Among the soil organisms actinomycetes are major producers of antibiotics especially *Streptomyces* genus. Among today's known antibiotics from microbes and comparable bioactive compounds (in total 22 500), 45% are produced from actinomycetes, 38% are produced from fungi and 17% are produced from unicellular bacteria. Although there are a wealth of compounds but only few hundred are in practically used for human treatment isolated from actinomycetes.[4] Actinomycetes considered as one of the richest prokaryotes that belong to the phylum Actinobacteria that have been considered accountable for the production of plenty of bioactive metabolites used as antibiotic and antitumor agents.[5] Chemical compounds discovered from actinomycetes such as antibiotic with more potent, cost effective and safer natural compounds.[6] Owing to the antibiotic potential of actinomycetes as noted in the above studies, the present work has aimed at exploring the antibiotic activity of the actinomycete population of rhizospeheric soil of Rajasthan, India.

Materials and Methods

Sample collection

Soil samples were combined together from different Rhizospheric soil of different locations of

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Rajasthan state. Collected soil samples were taken from locations like Udaipur, Kota, Alwar and Jaipur. To keep away from any contamination, rhizospheric soil was collected with clean, dry hand gloves in sterile air tight bags from mentioned four locations. From 12–15 cm deep soil samples were collected in bags and brought to laboratory for storage at 4°C for further processing of samples.[7]

Isolation of actinomycetes

From different locations of Rajasthan, these samples were brought in sterile polythene bags in laboratory. Purpose of isolation of actinomycetes, Actinomycetes isolation agar (AIA) media was used and subjected to dilutions upto 10^{-5} and pour plate and spread plates method were used for isolation of actinomycetes. Sprinkle method was also used for this purpose and plates were kept inverted at 37°C in incubator for 7–14 days. A total number of isolates was sixty five, isolated on actinomycetes isolation agar (AIA) media. Among all isolates some isolates were having potential activity against indicator pathogens. In the present study AIA26 isolate was further studied for their antibacterial activity. Isolate AIA26 was referred to tests such as gram staining and biochemical tests.[8]

Primary and secondary screening against indicator pathogens

Primary and secondary screening of isolated colonies was performed for detection of antimicrobial activity against five indicator pathogens. Disc diffusion and Agar well method was applied for primary screening of isolates.[7, 9]

Indicator pathogens for screening

Screening process of isolate AIA26 was executed by disc diffusion method and agar well diffusion method for the purpose of antimicrobial activity of isolates against some of the test pathogens spread onto Mueller Hinton Agar (MHA) media plates. Antimicrobial activity of isolate was performed against standard cultures of IMTECH Chandigarh. Five cultures were used namely *S. aureus* (MTCC-3160), *P. aeruginosa* (MTCC-1688), *K. pneumonia* (MTCC-432), *P. vulgaris* (MTCC-7306), and *B. subtilis* (MTCC-441). The inhibition activity was recorded as spread (mm) of inhibition zone (IZ).

Process of fermentation and centrifugation

For the antimicrobial compounds production by AIA26 isolate Luria broth was prepared. 500 mL of Luria broth was prepared and distributed into two 250 mL sterilized flasks. In each broth flask AIA26 was inoculated aseptically in laminar air flow hood and kept in shaker incubator at 150 Rpm at 30°C for around 21 days. Immediate after incubation is over, the cell suspension was centrifuged at 5000 Rpm for 15–20 min to separate the supernatant and the biomass. Supernatant was collected separately in a beaker and used for further tests.[10]

Solvent extraction process for bioactive compounds isolation

After fermentation, mycelium was removed from the fermented broth by filtration process and then clear filtrate was used for the detection of antimicrobial activity. Then the isolation of anti-microbial compound was done from the filtrate by solvent extraction method. Antimicrobial compounds were extracted from the filtrate by solvent extraction with Petroleum ether, Benzene, Ethyl acetate and Chloroform. Solvent extraction was followed with solvent mixed to the filtrate in 1:1 (v/v) ratio and shaken energetically for some time so that complete extraction of metabolites can be done. It was kept uninterrupted till 2 dissimilar layers get separated visibly. Two layers when separated collected in two beakers. Solvent was evaporated by keeping beakers on water bath at 50–60°C and crude remaining in beakers were measured and used for further testing.[11]

Antimicrobial activity of crude extract

After solvent extraction process with Petroleum ether, Benzene, Chloroform and Ethyl acetate solvent system, crude obtained from this was applied on test pathogens/indicator organisms already spread on the Mueller Hinton Agar (MHA) Petri plates. By the Disc diffusion method antibacterial activity test was carried out against above mentioned selected cultures. Plates were kept at 37°C for 24–48 hrs and results were observed and complete inhibition zone (IZ) was measured in millimeter (mm).[11]

Partial purification of compounds by Thin layer chromatography (TLC)

Prepared silica coated TLC plates were used for primary analysis of the antimicrobial substances. A dry crude benzene extract, dissolved in respective solvent, was spotted onto TLC plate and developed in the solvent system Hexane: Acetone (7:3, v/v). The developed TLC plates were air dried to remove all traces of solvents. For visualization Iodine chamber was prepared and plate was visualized. The retention factor (R_f) of the all four spots of AIA26 (Fig. 1, Table 1) benzene was measured. Total

Table 1 — Conditions and observations of Thin layer chromatography (TLC) of AIA26 isolate

Sample	Solvent system	Solvent ratio	R_f values	Color of spot	Spraying reagent	Uv visibility
AIA26 Ben	Hexane:Acetone	3:1	a=0.05,b=0.16, c=0.25, d=0.54	Dark brown	10% H_2SO_4	—
AIA26 Chl	n-butanol: Acetic acid:H_2O	4:1:5	a=0.45,b=0.54, c=0.74	Yellow	Iodine vapours	—

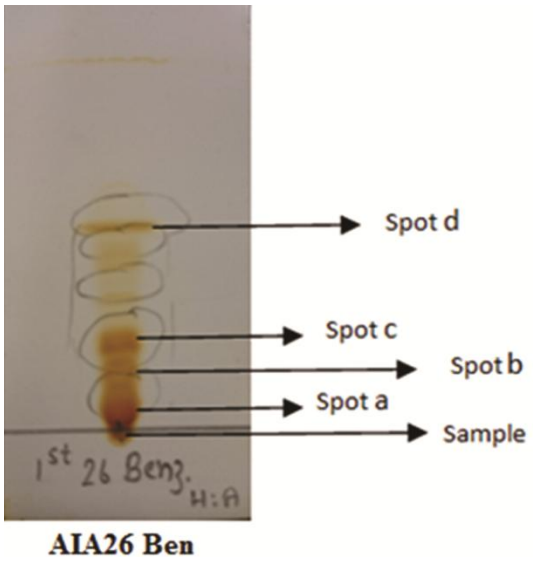

Fig. 1 — Separation of metabolites by Thin Layer Chromatography (TLC) of AIA26 Isolate

distance travelled by solvent was 5.5 cm. R_f value for spot a-0.054, spot b-0.163, spot c-0.25, spot d-0.54 was recorded.[12]

GC-MS Analysis

Compound extracts of AIA26 isolate were analyzed by GC-MS method. GC-MS analysis was executed using GC Shimadzu QP2010 ultra system and gas chromatograph interfaced to a Mass Spectrometer (GC-MS) and was operational with Elite-1 fused silica capillary column. Column-Rtx-Ms, 30 m × 0.25 mm i.d. × 0.25 μm film thickness was used for detection of sample. Samples were loaded in injector and processed. Chromatograms with compounds detected were recorded for each solvent of sample and compared with compound (NIST14. LIB) library Helium gas (99.99%) was the carrier gas with a constant flow rate of 1.21 mL/min and with split ratio: 10. Temperature of Injector was 260°C; Ion-source temperature 200°C. The oven temperature was intended from 60°C (constant for 3 min.) with an increment as of 280°C for 22 min. Mass spectra were taken at 70 eV with a scan interval of 0.5 sec.[13]

Results and Discussion

A total of sixty five isolates were recovered from rhizospheric samples. Isolates were sub-cultured and

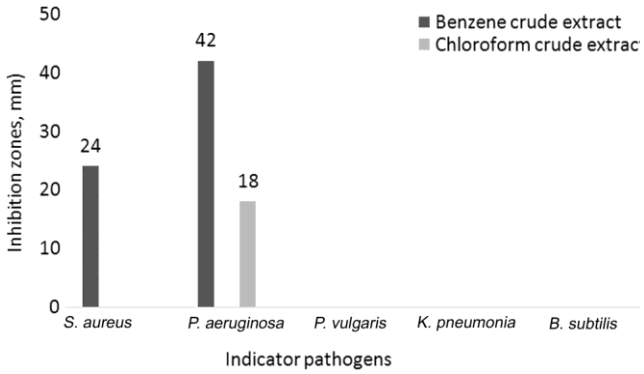

Fig. 2 — Antimicrobial activity of benzene and chloroform crude extracts of AIA26 isolate against standard Indicator pathogens

obtained pure colonies of isolates on AIA media from four selected locations of Rajasthan. In this study AIA26 isolate showed positive result testing for gram's staining followed by morphological, cultural and biochemical tests. Isolate showed susceptibility against selected indicator pathogens and demonstrated antimicrobial activity against indicator pathogens. Antagonistic activity tests were accomplished and positive results were showed by AIA26 isolate in primary screening.

Further the isolate was kept for process of fermentation for antimicrobial compounds production followed by solvent (benzene & chloroform) extraction of bioactive crude extracts of compounds present in the isolate. Indicator bacterial pathogens selected and used for the study were *S. aureus* (MTCC-3160), *P. aeruginosa* (MTCC-1688), *K. pneumonia* (MTCC-432), *P. vulgaris* (MTCC-7306), and *B. subtilis* (MTCC-441) for antibacterial activity test of isolate. Extracted crude of compounds from solvents were applied with sterile discs on prepared MHA plates containing lawn of bacterial strains. Inhibition zones (IZ) of antibacterial activity were recorded in mm. Activity of benzene crude extract of isolate was noted against two indicator pathogens as *S. aureus* and *P. aeruginosa* as presented in Fig. 2. Benzene extract of isolate was active against *S. aureus* and *P. aeruginosa* and inhibition activity was recorded as 24 mm 42 mm IZ respectively. Chloroform extract was active against *P. aeruginosa*, with IZ of 18 mm. Maximum activity was shown by crude extract of

benzene against *P. aeruginosa.* No activity was recorded against *K. pneumonia*, *P. vulgaris*, and *B. subtilis*.

Partial purification of crude extracts was performed using Thin Layer Chromatography (TLC) and observed four spots on TLC plate with different R_f values. Solvent system used as Hexane: Acetone (3:1) and n-butanol: Acetic acid: H_2O (4:1:5). R_f value for each spot was calculated; spot a-0.054, spot b-0.163, spot c-0.25, spot d-0.54. For visualization of plates Iodine chamber was used and then subsequent exposure to UV at 254 nm and at 365 nm. After these observations isolate was sent for GC-MS analysis for recognition of compounds present in sample. GC-MS technique uncovered the existence of numerous compounds in the isolate. The RT of each peak indicates different compounds present at different Retention time. Area of peaks is equal to the amount of compound present in the sample. Compounds present in sample were compared with two library; WILLEY 8 and NIST 14.LIB. Several compounds were detected in GC-MS analysis from benzene and chloroform extract. Some major ones are listed in Tables 2 & 3 with different RT.

Table 2 — Major compounds present in benzene extract of AIA26 isolate detected in GC-MS analysis

Name of the Compounds	RT, min
Cyclopropanecarbox amide, n-(2-octyl)	11.495
(3s,3as,7r)-3a,4,5,6,7a-tetrahydro-3,6-dimethylbenzofuran-2(3h)-one	15.487
1-hexadecene	16.289
Nonane, 3,7-dimethyl-	16.456
Undecane, 3,8-dimethyl-	18.942
Sulfurous acid, 2-ethylhexyl isohexyl ester	20.828
L-proline, n-valeryl-, undecyl ester	21.763
1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	22.334
Hexane, 2,4,4-trimethyl-	22.875
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	23.823
1,2-benzenedicarboxylic acid, butyl 8-methylnonyl ester	24.212
1-nonadecene	24.718
Sulfurous acid, 2-ethylhexyl hexyl ester	24.833
Decane, 3,8-dimethyl-	26.018
Nonane, 5-methyl-5-propyl-	27.432
Behenic alcohol	28.407
Ergotamine	30.954
Ethylcyclodocosane	31.797

Table 3 — Total area %, formula, and weight of compounds present in both benzene and chloroform crude extracts of AIA26 isolate detected at different RT in GC-MS analysis

Name of the Compounds	% in Benzene extract	RT, min	% in Chloroform extract	RT, min	Total %	Mol. formula	Mol. Wt
5,9-dimethyl-2-(1-methylethylidene) cyclodecanone	18.13	29.508	—	—	18.13	$C_{15}H_{26}O$	222
1,2-benzenedicarboxylic acid	9.33	34.7	1.3	34.742	10.63	$C_{24}H_{38}O_4$	390
1,4-epoxynaphthalene-1(2h)-methanol, 4,5,7-tris(1,1-dimethylethyl)-3,4-dihydro-	9.08	41.925	—	—	9.08	$C_{23}H_{36}O_2$	344
2-cyclopenten-1-one, 2-hydroxy-3-methyl-	7.87	20.099	—	—	7.87	$C_6H_8O_2$	112
Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, octadecyl ester	7.84	41.598	—	—	7.84	$C_{35}H_{62}O_3$	530
1-hexadecene	3.19	16.289	—	—	3.19	$C_{16}H_{32}$	224
1-octanol	2.3	13.472	—	—	2.3	$C_8H_{18}O$	130
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	2.29	21.249	40.9	21.939	43.19	$C_{11}H_{18}N_2O_2$	210
Acetamide, n-[2-(1h-indol-3-yl)ethyl]-	—	—	7.45	29.21	7.45	$C_{12}H_{14}N_2O$	202
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenyl methyl)	—	—	5.83	31.755	5.83	$C_{14}H_{16}N_2O_2$	244
1-heptacosanol	—	—	4.6	31.872	4.6	$C_{27}H_{56}O$	396
O o'-biphenol, 4,4',6,6'-tetra-t-butyl-	—	—	4.09	31.541	4.09	$C_{28}H_{42}O_2$	410
N-tetracosanol-1	—	—	3.87	28.463	3.87	$C_{24}H_{50}O$	354
3-methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-dione, n-acetyl	—	—	3.54	19.539	3.54	$C_{10}H_{14}N_2O_3$	210
5h,10h-dipyrrolo[1,2-a:1',2'-d]pyrazine-5,10-dione, octahydro-, (5as-cis)-	—	—	2.74	24.121	2.74	$C_{10}H_{14}N_2O_2$	194
Phenol, 2,4-bis(1,1-dimethylethyl)-	—	—	2.64	14.54	2.64	$C_{14}H_{22}O$	206
Docosyl heptafluorobutyrate	—	—	2.05	39.83	2.05	$C_{26}H_{45}F_7O_2$	522

Biological activities of some compounds such as 1-heptacosanol has been reported as anti-bacterial, nematicidal, anticancer, anti-oxidant and anti-microbial compound.[14] Phenol, 2,4-bis(1,1-dimethylethyl)- has anti-bacterial, and anti-inflammatory properties.[15] 1-hexadecene has antibacterial, antifungal, and antioxidant activities.[16] Compound Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- has been shown to have both anti-bacterial and anti-fungal activity isolated from *Streptomyces* species.[17] Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, octadecyl ester has been reported as antifungal and antioxidant compound.[18] The biological activity of many compounds have not been studied yet including 3-methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-dione, n-acetyl, 2-cyclopenten-1-one, 2-hydroxy-3-methyl-, and 1,4-epoxynaphthalene-1(2h)-methanol, 4,5,7-tris(1,1-dimethylethyl)-3,4-dihydro-, and many other compounds.

Conclusions

Numerous compounds at different Retention time detected from GC-MS analysis possess Antibacterial activity. The present study indicates that the soil samples of Rajasthan have potential antimicrobial activity to fight against indicator pathogens. The extra-cellular compounds retrieved from solvents displayed antibacterial activity against two pathogens *S. aureus* and *P. aeruginosa*. GC-MS analysis of the extracts directed, Pyrrolo [1,2-a]pyrazine-1,4-dione hexahydro-3-(2-methylpropyl) as a main component (43.19%) present in the isolate. Overall study was successful in finding antimicrobial components from the rhizospheric soil and to continue the search for more antibiotics or antimicrobial components from these unexplored locations of Rajasthan.

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