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SECONDARY METABOLITES OF ACTINOMYCETES AND THEIR ACTIVITY AGAINST PSEUDOMONAS AERUGINOSA

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Abstract

Actinomycetes are widespread in ecosystem and they are of interest in research field as they are capable to produce bioactive secondary metabolites which canbe developed into useful pharmaceutical products. The potential of antibacterial activity of derived actinomycetes from the genus Streptomyces were studied. The actinomycetes were identified as putative Streptomyces spp. and 4 strains were selected. Selected putative strains of Streptomyces spp. for bioactivity screening on inorganic salts starch agar (ISP4) for strains T3 and T4 and sporulation agar (SA) for the remaining strains. Inorganic salts-starch agar (ISP4) showed grey coloured spores and sporulation agar (SA) showed White/Brown spores.In primary screening, nine strains showed antagonistic activity against at least one test pathogenic bacteria. In secondary screening, all strains showed antagonistic activity against at least one test pathogenic bacteria. The culture filtrate extracted with ethyl acetate by adjusting the pH to 3 prior to extraction process resulted in maximum recovery of antimicrobial metabolites. The ethyl acetate crude extract obtained from the TC1 fermentation broth exhibited a MIC value of 300 µg/mL and IC 50 value of 125 µg/mL against the test pathogen P.aeruginosa.The culture filtrate and crude extract of Streptomyces sp. TC1 showed antimicrobial activity against various isolates of P.aeruginosa.Thin layer chromatographic separation of ethyl acetate crude extract of Streptomyces sp. TC1 resulted in nine bands with closer Rf values using chloroform: methanol (24:1 v/v) as the mobile phase. Consequently antimicrobial compounds were purified using column chromatography. Bioactivity guided column chromatographic separation of ethyl acetate crude extract afforded 27 different fractions using n-hexane-chloroform as a mobile phase. Among 27 different fractions, four fractions showed antimicrobial activity against Xoo by producing an inhibition zone of 1.9- 2.5 cm in agar well diffusion assay. The fraction F4 exhibited higher antimicrobial activity of 2.5 cm followed by F15, F9 and F22 which produced inhibition zone of 2.3, 2.0 and 1.9 cm respectively.The isolated antimicrobial metabolites including three novel compounds viz., 2- Clavulanic acid (compound 1) and 2-(1,1-diallyl-but-3-enyl)-5-nonyl-phenol (compound 2).

Keywords: Pseudomonas, Cetrimide agar, Actinomycetes**, S**treptomyces**,** MIC, Opportunistic Pathogen.

INTRODUCTION

Actinomycetes are filamentous Gram-positive bacteria, characterized by a complex life cycle belonging to the phylum Actinobacteria, which represents one of the largest taxonomic units among the 18 major lineages currently recognized within the Domain Bacteria (Vance et al., 2005). The bioactive secondary metabolites produced by microorganisms is reported to be round 23,000 of which 10,000 are produced by actinomycetes, thus representing 45% of all bioactive microbial metabolites discovered (Solans, and Vobis, 2003). Among actinomycetes, approximately 7,600 compounds are produced by Streptomyces species (Prashith Kekuda et al., 2010). Several of these secondary metabolites are potent antibiotics.

Bioactive metabolites are products of primary and secondary metabolism of different organisms (plants, animals, fungi, bacteria). They often demonstrate biological activity (Demain A.L., Sanchez, 2009). Secondary metabolites have diverse and unusual chemical structures, and often a low molecular mass (Donadio et al., 2002 and Bérdy, 2005). Secondary metabolites, unlike primary metabolites, have no function in the life cycle of cells and are characterize specific groups of organisms. The production of secondary metabolites from the genus Streptomyces can be influenced by optimization of the nutritional requirements and cultural conditions. These conditions play an important role in the production of these secondary metabolites (Al-Hulu, 2013).

MATERIALS AND METHODS

Sample Collection

The actinomycetes used in this study were obtained from Prof. Dr.Vikineswary Sabaratnam from Plant Mycology and Pathology Laboratory, Institute of Postgraduate Studies, University of Malaya (IPS, UM). The actinomycetes were identified as putative Streptomyces spp. and 4 strains were selected for further studies. Pure cultures of actinomycetes strains could be prepared using the stock cultures.

Isolation and Characterization of Pseudomonas aeruginosa

Clinical samples such as urine were collected from the microbiology laboratory of Royal care Hospital, Coimbatore District, Tamilnadu, India. The urine (mid-stream urine 146 numbers) were analysed by direct gram staining and culturing the sample on nutrient agar, blood agar and MacConkey agar (CLSI,2011). The inoculated plates were incubated at 370C for 24 hours. The characteristic isolates were identified by confirmative test widely practiced in clinical laboratories.

Screening for Secondary Metabolite Production

a. Primary Screening

Cross streak method was done by a vertical single line streak of 72 hours actinomycete broth culture through the center of muller hinton agar (Himedia, India) plates and incubated at 280C for four days. 12 hours young bacterial culture was cross streaked horizontally as single line against actinomycete growth on the plates (BSAC, 2010).

b. Secondary Screening

Disc Diffusion Method

Kirby - Bauer disc diffusion method was employed for testing bioactivity. 12 hours young Pseudomonas aeruginosa culture was effectively swabbed on muller hinton agar plates. The plates were kept undisturbed for 20 minutes in upright position. The culture filtrate loaded filter discs were placed aseptically on inoculated agar surface using a disc dispenser or a sterile forceps. The plates were incubated at 37^0C for 24 hours. After incubation the plates were observed and the zone of growth inhibition around the discs was measured using a millimeter ruler from the underside of the plate.

Antimicrobial activity assay

A quantity of 10 mg of crude extract obtained from solvent extraction of TC1 culture filtrate were dissolved in 1 mL of 100 percent ethanol and used for antibacterial 37 assays against the P.aeruginosa isolates by agar well diffusion method (Bauer et al.,1996).

Separation of secondary metabolites through thin layer chromatography (TLC)

To visualize the number of compounds present in the extract of isolates (PS1 and PS28), thin layer chromatog-raphy (TLC) was performed. Aluminum plates pre-coated with silica gel (20×20 cm, 0.25 mm Alugram® SIL G/UV 254, Macherey and Nagel, Duren) and two mobile phases [ethyl acetate : methanol (6:4) and Petroleum ether : Chloroform (1:1)] (Attimarad et al., 2012). Chromatograms were observed under UV light, frac-tions of the isolates retention factor Rf value were measured and the ratio calculated.

Separation of secondary metabolites using column chromatography

The separation and purification of antimicrobial compounds from the ethyl acetate crude extract of Streptomyces sp. TC1 were carried out using silica gel column chromatographic technique.

RESULTS

During this study period 146 samples were received, from these samples 69 bacterial isolates were obtained. Out of 69 isolates 16 were identified as Pseudomonas species. Out of this predominant numbers were that of P.aeruginosa 16 (84.25%), followed by other Pseudomonad's 3 (15.75%). Majority of the isolates of P.aeruginosa were isolated from urinary samples. P. aeruginosa was mainly isolated from cultures (Fig-1).

Isolation and inoculation of actinomycetes

All the plates were observed with dried powdery shape colonies after an incubation of five days. The distinct colonies were taken using a sterile loop and are subcultured onto SCA slants. A total of 16 colonies were isolated from different samples and sub-cultured. Selected putative strains of Streptomyces spp. for bioactivity screening on inorganic salts-starch agar (ISP4) for strains T3 and T4 and sporulation agar (SA) for the remaining strains. Inorganic salts-

starch agar **(**ISP4) showed grey coloured spores and sporulation agar (SA) showed White/Brown spores (Fig-2).

Antibacterial pattern in primary and secondary screening

The number of active Streptomyces spp. against test bacteria P.aeruginosa was higher in secondary screening compared to primary screening. However, the number of active strains against test bacteria P.aeruginosa was higher in secondary screening compared to primary screening.

In primary screening, nine strains showed antagonistic activity against at least one test pathogenic bacteria (Fig-3). In secondary screening, all strains showed antagonistic activity against at least one test pathogenic bacteria (Fig-4).

Antimicrobial activity of culture filtrate and crude extract against various isolates of Streptomyces

The antimicrobial activity in terms of zone of inhibition against various isolates of P.aeruginosa was carried out for the TC1 culture filtrate as well as crude extract. The crude extract produced a zone of inhibition of 1.5-2.2 cm against the tested P.aeruginosa isolates. The highest activity of 2.2 cm inhibition zone was found against the P.aeruginosa isolate obtained from TN1 variety, whereas the lowest activity of 1.5 cm zone of inhibition was observed for P.aeruginosa isolate obtained from CO50 variety. The ethyl acetate crude extract exhibited higher antimicrobial activity compared to culture filtrate which produced an inhibition zone of 1.1-1.6 cm against the tested isolates. The variation observed in the antimicrobial activity against the P.aeruginosa pathogen is due to the existence of different resistance genes among the races of P.aeruginosa isolates (Table-1).

Thin layer chromatography (TLC) separation of crude extract

TLC is a technique for separation of bioactive fractions from the crude extracts of microbial and plant origin. The TLC separation of ethyl acetate crude extract was carried out for the separation of antimicrobial compounds using different solvents as mobile phase. The better separation of crude extract was detected in chloroform: methanol (24:1v/v) as a mobile phase. A total of 9 bands were observed for ethyl acetate crude extract, which was observed under visible, UV light and iodine vapours (Fig-5).

The Rf value of the separated bands was observed very closer. Hence, TLC is not considered as an appropriate method for separation of compounds in pure form. Further the column chromatography was preferred to purify the antimicrobial metabolites from ethyl acetate crude extract.

Fractionation of ethyl acetate crude extracts using column chromatography and its antimicrobial activity

Column chromatography is a technique used to purify individual chemical compounds from mixture of compounds, which is a prerequisite for structural elucidation and bioactivity evaluation. Column chromatographic studies were carried out for the purification of active fraction from ethyl acetate crude extract obtained from culture filtrate of Streptomyces sp. TC1.

The mass production of Streptomyces sp. TC1 was carried out in 1000 mL of production medium for the extraction of crude fractions. The crude extract of 2.3 g was obtained from the 25 L of fermentation broth. The obtained crude extract was subjected to column chromatography over silica gel (60-120 mesh) with the gradient elution of n-hexane-CHCl3. The polarity of n-hexane gradually increased using chloroform upto 15:85 v/v (n-hexane: chloroform) to give 275 fractions of 100 mL each. Fractions with similar TLC chromatogram were pooled together and as a result total of 27 different fractions were obtained which were labeled as F1-F27. The fractions were tested for their antimicrobial activity against P.aeruginosa by agar well diffusions method. Among the collected fraction F4 exhibited higher antimicrobial activity against P.aeruginosa (2.5 cm) followed by F15 (2.3 cm), F9 (2.0 cm) and F22 (1.9 cm) (Table-2).

Column chromatographic elution details of antimicrobial fractions

The fractions obtained with the mixture of n-hexane-CHCl3 (88:12 v/v) yielded compound 1 (F4). Further a gradient elution using a mixture of n-hexane-CHCl3 (46:54 v/v) yielded compound 2 (F9) as white crystalline in nature. The above said fractions exhibiting antimicrobial activity against P.aeruginosa were checked for their purity in TLC and subjected to spectroscopic studies for their structural elucidation.

Analysis of functional groups of isolated compounds by TLC spray reagents

TLC spray reagents were used to find out the functional groups present in the isolated antimicrobial compounds as well as in crude extract. The spray reagents indicate the presence of clavulanic acid and phenolic components in the crude extract. The appearance of blue colour spot with Folins Ciocalteu reagent confirms the presence of phenolic functional group in compound **1**. The development of spots under UV light after spraying with ethanolic solution of KOH (5%) denotes the presence of clavulanic acid moiety in compound 2 (Table-3).

Antimicrobial activity of isolated compounds 1 and 2 against various isolates of Streptomyces

The antimicrobial activity in terms of zone of inhibition against various isolates of Streptomyces was carried out for the isolated compounds and the results were tabulated (Table-6). The compound 1 produced a zone of inhibition of 1.8-2.5 cm against the tested Streptomyces isolates. The highest activity of 2.5 cm inhibition zone was found against the Streptomyces isolate obtained from TN1 variety. Whereas the lowest activity of 1.8 cm zone of inhibition was observed for Streptomyces isolate obtained from CO50 variety. The compound 1 produced an antimicrobial activity in the range of 1.5-2.0 cm against Streptomyces isolates. Whereas compound 2 exhibited antimicrobial activity of 1.7-2.3 cm against the tested isolates.

DISCUSSION

The resistance of numerous pathogenic bacteria to antibiotics necessitate search for new antibacterial agents to fight these pathogens. Secondary metabolites produced by bacteria still interested, due to their complicated chemical structures and highly specific antimicrobial activities. The soil bacteria resembling to the genus Streptomyces are rich sources of large number of bioactive natural products; they are widely used as antimicrobials. Streptomyces species produce about 75% of useful antibiotics (Kumar et al., 2014). Al-Zahrani, (2007)

reported that incubation conditions influence the qualitative production of secondary metabolites by Streptomyces species. Kathiresan et al., (2005) reported that ability of bacteria to produce antimicrobial agents can be increased or lost under different culture conditions. Maximum antibiotic production was observed with soyabean meal as a source of nitrogen compared to other compounds.

Our result shared by all the isolates including the reference strain P. aeruginosa PAO1 is the positive reaction for oxidase. This is due to the presence of cytochrome coxidase in the electron transport chain. Cytochrome oxidase is an enzyme found in some bacteria that transfers electrons to oxygen, the final acceptor in some electron transport chains (Miller et al., 1979). This is one of the distinguishing characteristics that differentiate the pseudomonads from enteric bacteria (Hampton and Wasilauskas, 1979).All of the isolates were also catalase positive, which is typical of Pseudomonas strains (Bergey's Manual for Systematic Bacteriology, 2001).

An antagonistic strain TC1 against P.aeruginosa was screened and identified as Streptomyces sp. by morphological characterization and biochemical assays and was further confirmed by comparison of its 16S rRNA gene sequence. The comparative BLAST analysis revealed that the TC1 was found to be 96% similar to that of Streptomyces sp. DL-28 and has been deposited in GenBank with accession number 205 KC954629 (Tahvonen, 1982b).

The bioactive fractions from fermentation broth were usually separated by extraction with suitable organic solvents. The extraction using organic solvents was mostly carried out by the methods proposed by Westley et al. (1979) and Li et al. (2002). Zhang and Sherman (2001) reported the isolation of a novel macrolide antibiotic novamethymycin from the culture filtrate of Streptomyces venezuelae. The antimicrobial compound isolated by silica gel column chromatography using the ethyl acetate crude extract of culture filtrate was used for structural elucidation by spectroscopic techniques. The isolated novamethymycin produced an inhibition zone of 1.8 cm against Bacillus subtilis in disk diffusion assay.

The antimicrobial compounds isolated from the culture filtrate of Streptomyces sp. PM5 showed to be promising for control of major rice pathogens, Pyricularia oryzae and Rhizoctonia solani both under in vitro and in vivo conditions. The isolated antimicrobial metabolites SPM5C-1 and SPM5C-2 belonging to aliphatic group containing lactone and ketone carbonyl chains respectively, were fully characterized by using spectroscopic techniques like GC-MS and NMR (Prabavathy et al., 2006).

The antimicrobial activity of compound 2 was supported by the antibiotic action of propionic acid against many gram positive and gram negative bacteria (Thompson and Hinton, 1997). Goodfellow and Williams (1983) reported that introducing fluorine atoms in short cationic peptides further enhanced its antimicrobial activity. Thus the isolated compound 2 exhibited antimicrobial activity by having propionic acid moiety and fluorine atom in its chemical structure. Many of these compounds are responsible for the antibacterial activity that detected in this study.

CONCLUSION

The study concluded that marine is promising habitat Streptomyces spp. and optimizing of culture conditions may influence the production of secondary metabolites. Based on the results the study reached a conclusion that Streptomyces sps. can be used for the development of drugs against bacterial pathogens.

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Table-1:Antimicrobial activity of isolated compounds against P.aeruginosa

Source of Streptomyces isolate	**Diameter of inhibition zone (in cm)**
TN 1	2.5 ± (0.12)
TNRH 180	2.2 ± (0.10)
CO 47	2.1 ± (0.06)
CO 50	1.8 ± (0.15)
ADT 39	2.2 ± (0.06)
ADT 43	2.0 ± (0.12)

SEd	0.086
CD(.05)	0.188

Values are mean ± SD of three replicates

Table-3: Detection of chemical groups using spray reagents by TLC

Sl. No.	Test	Result
1.	Alkaloids	-
2.	Coumarins	+
3.	Flavonoids	-
4.	Phenols	+
5.	Saponins	-
6.	Steroids	-
7.	Tannins	-
8.	Terpenoids	-

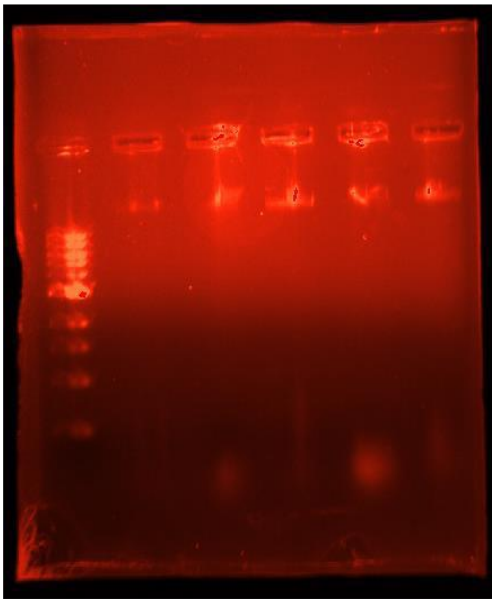
Table-2:Antimicrobial activity of the fractions separated from column chromatography

Fraction No.	DIZ (in cm)
F1	-
F2	-
F3	-
F4	2.5 ± (0.12)
F5	-
F6	-
F7	-
F8	-
F9	2.0 ± (0.06)
F10	-
F11	-
F12	-
F13	-
F14	-
F15	2.3 ± (0.06)
F16	-
F17	-
F18	-
F19	-
F20	-
F21	-
F22	1.9 ± (0.15)
F23	-

F24	-
F25	-
F26	-
F27	-
NC	0.1 ± (0.06)
PC	3.2 ± (0.06)

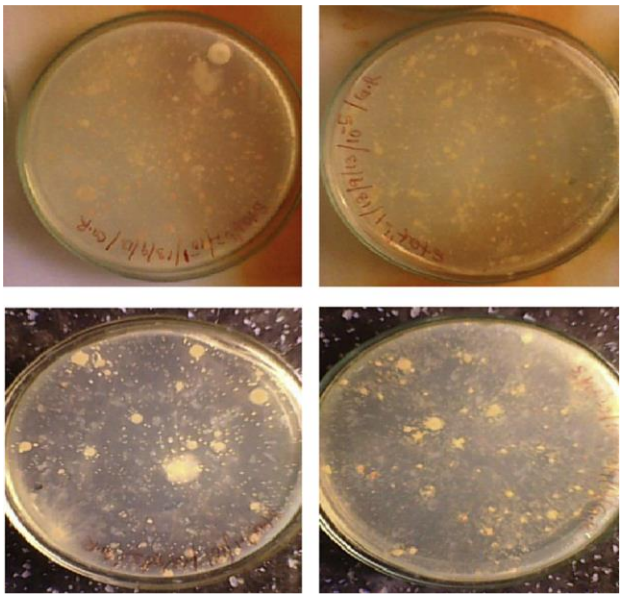
Values are mean ± SD of three replicates

Fig-1:Microscopically examination of pseudomonas aeruginosa



Microscopically pseudomonas aeruginosa are motile (one or more polar flagella), rod shaped and aerobic, Gram-negative, non-fermentative bacteria. The typical bacterial size is 0.5 – 1.0 x 1.5 – 5.0 μm.

Fig-2: Isolation of actinomycetes



Cultures of selected putative strains of Streptomyces spp. for bioactivity screening incubated at 28±2oC for 7 – 14 days on ISP4 for strains T3 and T4 and SA for the remaining strains

Fig-3: **Primary screening of Streptomyces sp.**

Fig-4: Secondary screening of Streptomyces sp.

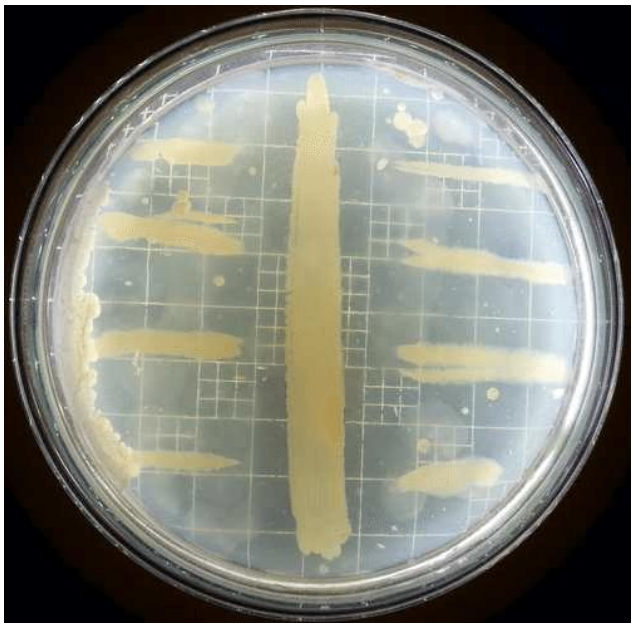


Fig-5: Thin layer chromatographic (TLC) separation of ethyl acetate crude extract and isolated compounds

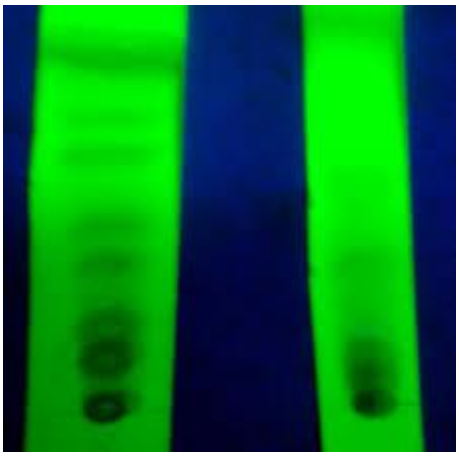


Fig-6: **Antibacterial effect of Actinomycetes against Pseudomonas aeruginosa**