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ISOLATION, CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF ACTINOBACTERIA FROM DYE POLLUTED SOILS OF TIRUPUR

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Abstract. *The study revealed that the 31 actinobacterial isolates were isolated from dye polluted soils. The 31 actinobacterial isolates were grouped into 6 genera, among which Streptomyces was the predominant genera. Actinobacterial isolates were screened for their antibacterial properties. Only 2 isolates, namely RMS3 and RMS6, showed promising antibacterial activity against bacterial pathogens. The antibacterial activity of the antagonistic actinobacteria RMS3 and RMS6 showed maximum on Bacillus subtilis and Klebsiella pneumoniae. The potent actinobacteria were identified as Streptomyces sp. RMS3 and Nocordia sp. RMS6 on the basis of their phenotypic properties, and their antibacterial compound was similar to cephalexin and spiramycin respectively*.

Key words: *Actinobacteria, textile effluent polluted soil, antibacterial activity, HPLC*

Introduction

Actinobacteria are the most widely distributed group of microorganisms in nature. They are attractive, bodacious filamentous Gram positive bacteria having high GC content in their DNA. Actinobacteria are considered to be an intermediate group between bacteria and fungi. Majority of actinobacteria are free living, saprophyte found in soil, water and colonizing in plants. Actinobacteria are noteworthy as antibiotic producers, making three quarters of all known products; especially streptomycetes produced many antibiotics and other class of biologically active secondary metabolites, they cover around 80% of total antibiotic product, with other genera. It is anticipated that the isolation, characterization and the study on actinobacteria can be useful in the discovery of antibiotics from novel species of actinobacteria [1].

Streptomycetes is the largest antibiotic producing genus in the microbial world. The number of antimicrobial compounds reported from streptomycetes has increased almost exponentially in the last two decades. About 4,000 antibiotic substances have been discovered from bacteria and fungi, many of them are produced by streptomycetes. Most of the streptomycetes produce a diverse array of antibiotics including aminoglycosides, anthracyclins, glycopeptides, polyether and tetracycline [2]. Screening of microorganisms for the production of novel antibiotics has been intensively pursued for many years. Antibiotics have been used in many fields including, veterinary and pharmaceutical industry. Actinobacteria have the capability to synthesize many different biologically active secondary metabolites such as antibiotics, herbicides, pesticides, anti-parasitic compounds and enzymes like cellulose and xylanase used in waste treatment [3].

The abundance of terrestrial actinobacteria and their antibiotic productivity are known. The terrestrial actinobacteria would be an important source for the discovery of new antibiotics. Unfortunately, the rate of discovery of new compounds from existing genera obtained from terrestrial sources has decreased, while the rate of re-isolation of known compounds has decreased. Moreover, the rise in the number of drug-resistant pathogens and the limited success of strategies in proceedings new agents indicate an uncertain forecast for future antimicrobial therapy [4,5]. Thus, it has been emphasized that new group of microbe from unexplored habits be pursed as sources of novel antibiotics and other small therapeutic agents [6]. The perusal of the literature proved that there are not many reports of actinobacteria from textile effluent polluted soils. Keeping these points in view, the present study has been undertaken to isolate and screen the antibiotic producing actinobacteria from dye polluted soils of Tirupur, Tamil Nadu. Further, the identified antagonistic actinobacteria were characterized based on morphological, biochemical, cultural and physiological characteristics.

Material and Methods

Collection of soil sample. The soil samples were collected from textile dye polluted areas of Tirupur. The top layer soil samples were collected aseptically in ster-

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ile polythene bags. Samples were brought to the laboratory and stored at 4ºC for further assay.

Isolation of actinobacteria**.** Starch casein nitrate (SCN) agar medium (Himedia, Mumbai) was used for isolation and enumeration of actinobacteria. The medium was supplemented with 10 µg/ml amphotericin and 25 µg/ml streptomycin (Himedia, Mumbai) to prevent fungal and bacterial contamination respectively. Using conventional dilution plate technique, 10g of soil samples were suspended in 100 ml of distilled water and 0.5 ml of suspension from this was spread over starch casein agar medium and incubated for 7–9 days at 28ºC. After incubation, the actinobacterial colonies were purified and sub-cultured on SCN agar plates and stored for further assay.

Screening for antibacterial activity. Antibacterial activities of isolates were tested preliminarily by cross streak method [7]. Actinobacteria isolates were streaked across diameter on starch casein agar plates. After incubation at 28ºC for 6 days, 24 h cultures of *Bacillus subtilis* and *Klebsiella pneumoniae* were streaked perpendicular to the central strip of actinobacteria culture. All plates were again incubated at 30ºC for 24 h and zone of inhibition was measured.

Characterization of antibacterial compounds

Extraction of antibacterial compounds**.** The antagonistic actinobacteria RMS3 and isolate RMS were inoculated into starch casein broth. They were then incubated at 28ºC for 10 days in a shaker at 200-250 rpm. After incubation the culture filtrate was obtained by filtering through Whatmann No.1 filter paper and Millipore filter (Millipore Millex-HV Hydrophilic PVDF 0.45 µm). To the culture filtrates, equal volume of solvents (ethyl acetate, acetone, butanol, chloroform and distilled water) was added and centrifuged at 5000 rpm for 10 min to extract the compounds [8]. The compounds obtained from different solvents were tested for their antibacterial activity against the test organisms (*Bacillus subtilis* and *Klebsiella pneumoniae*) by 'well diffusion' method. The lawn cultures of bacteria on the Muller-Hinton agar plates were prepared. The 5 mm diameter well was made using sterile cork borer. The mixture (solvent and antibacterial compound) was poured into the well separately and incubated at 37ºC for 24 h. The solvents alone were used as control. After incubation the zone of inhibition was measured. The maximum antibacterial effect shown in solvent extracts were selected for further assay.

Antibacterial compound analysis by HPLC. The chromatographic separation of antibacterial compound was carried out on a LC-10 AT vp model HPLC using 250 x 4.60 mm Rheodysne column (C-18). The solvent system used was methanol (HPLC grade) and water (HPLC grade) in the ratio of 88:12. The operating pressure was 114 kgf, at a flow rate 0.8 ml/min and the temperature was set at 30ºC. The UV-Vis (SPD-10 A vp) detector was set at 210 nm. The sample was mixed with the solvent in the ratio of 50:50 and filtered using Millipore filter before injection. About 25 µL of the sample filtrate was injected into the column. The sample was run for 10 min. and the retention time was noted. The elution time was compared with the standard and the compound was determined [9,10].

Characterization of actinobacteria. Colony morphology of actinobacteria were recorded with respective colour of aerial and substrate mycelium, size and nature of colonies reverse side colour and pigmentation on starch casein agar medium as recommended by International *Streptomyces* Project (ISP) [11]. Microscopic characterization was carried out by cover slip culture method [12]. Actinobacteria culture plates were prepared on starch casein agar medium and 5-6 sterile cover slips were inserted at an angle of 45º. The plates were incubated at 28ºC for 4–8 days. The cover slips were removed and observed under high power magnification. The morphological features of spores, sporangia and aerial and substrate mycelia were observed. Actinobacteria were identified using standard manuals (Bergey's Manual of Systematic Bacteriology and Bergey's Manual of Determinative Bacteriology). The formation of aerial and substrate mycelia and arrangement of spores on mycelium were observed under high power objective of light microscope. Cultural characteristics (growth, colouration of aerial and substrate mycelia, formation of soluble pigment) were tested. Actinobacterial isolates were inoculated onto different media, starch casein agar, nutrient agar, yeast extract malt extract agar (ISP2), oat meal agar (ISP3), inorganic salt agar (ISP4), glycerol asparagine agar (ISP5). The plates were incubated at 28ºC for 7 days. After incubation the colony morphology with respect to colour, aerial mycelium, size and nature of colony, reverse side colour and pigmentation on different media were recorded.

Biochemical tests including H_2S production, catalase, oxidase, urease, nitrate reduction, starch, lipid, gelatine and casein hydrolysis, haemolysis, melanin pigment production and triple sugar iron (TSI) were also performed as recommended by ISP. Chemo-taxonomical properties, such as analysis of whole cell sugars [13] and cell wall amino acid analysis [14] were analyzed. Physiological characterization, such as the effect of pH (3–11), temperature (10–50ºC) and salinity (NaCl concentrations 1–16%) and antibiotic sensitivity against ten different antibiotics (Himedia, Mumbai) [cloxacillin, amikacin, ampicillin, tobramycin, ciprofloxacin, nitrofurantoin, nalidixic acid, trimethrioprim, streptomycin, tetracycline and trimethoprin] were also tested. Utilization of carbon sources, such as starch, dextrose, fructose, maltose and mannitol, and nitrogen sources namely L-arginine, L-aspargine, L-cystine, L-histidine and L-tyrosine were tested on starch casein agar medium.

Results

A total of 297 actinobacterial colonies were isolates from 25 dye polluted soil samples of Tirupur, India.

Among 297 colonies, based on the actinobacteria colony morphology, 31 morphologically distinct isolates were purified, sub cultured and maintained on cultivation medium (SCA) for further characterization. The actinobacterial isolates showed a distinguished array of macroscopic features such as aerial and substrate mycelium and diffusible extra cellular pigments. These isolates formed white and yellow coloured aerial mycelium, reverse side colour was yellow, white and dark yellow.

Under light microscope, actinobacterial isolates showed formation of aerial and substrate mycelium, spore mycelium and various structures like spiral, filamentous, spirally twisted and elongated aerial mycelium. Based on the colony morphology and microscopic characterization, the actinobacteria were identified to generic level. Among 31 isolates, 21 (68%) isolates belonged to the genus *Streptomyces*, *Nocordia* 3 (10%), *Actinopolyspora* 3 (10%), *Kitasatosporia* 2 (6%), *Catellospora* 1 (3%) and *Glycomyces* 1 (3%). Among the genera recorded, *Streptomyces* was the most predominant and frequently occurred in soil when compared to other genera.

All the 31 isolates were screened for their antibacterial activity against *Bacillus subtilis* and *Klebsiella pneumoniae*. Among 31 isolates, 17 (54.84%) isolates showed activity against test bacteria. All the 17 isolates showed activity on Gram-positive bacteria, whereas seven isolates inhibited Gram-negative bacterial growth, and 7 isolates had both Gram positive and negative bacteria. The isolates which possessed strong antibacterial activity against both Gram positive and negative were selected for further study. Culture filtrates of 7 isolates (both Gram positive and Gram negative activity) were tested for their antibacterial activity by well diffusion method. Among the 7 isolates only 2 showed maximum activity against all the pathogens tested. Hence, these two potent isolates were selected for further characterization.

The antibacterial activities of the actinobacterial extracts were tested against two bacteria. The maximum inhibition effect was showed with ethyl acetate extract of *Streptomyces* sp. RMS3 against *K. pneumoniae* (26 mm) and *B. subtilis* (22 mm). Similarly, the maximum antibacterial activity of *Nocardia* sp. RMS6 was showed with ethyl acetate extract against *B. subtilis* (30 mm) and *K. pneumoniae* (26 mm). Further, methanolic extracts of both isolates showed maximum activity, whereas other solvent extracts showed moderate to minimum inhibition effect against all the pathogens tested (Table 1).

The antibacterial compounds were analyzed by HPLC analysis. The absorption peak values of *Streptomyces* sp. RMS3 compound showed at 3.587 and 6.520 min. whereas the compound of *Nocardia* sp. RMS6 showed absorption peaks at 3.677 and 6.573 min. Both antibacterial compounds showed single major compound. On the basis of retention time and absorption peaks the antibacterial compounds were compared with the standard antibacterial compounds. Retention time of RMS3 compound was found similar to cephalexin, when compared with the HPLC pattern of standard antibacterial compound (Fig. 1). Similarly, retention time of RMS6 compound was found to be spiramycin, when compared with the HPLC pattern of standard antibacte-

Table 1. Antibacterial efficacy of actinobacteria

Name of the Strain	Name of the pathogen	Zone of inhibition (mm)				
		Alcohol	Chloroform	Distilled water	Ethyl acetate	Methanol
Streptomyces sp. RMS3	*B. subtilis*	14 (4)	12 (7)	12 (0)	22 (8)	14 (5)
	K. pneumoniae	13 (5)	14 (3)	13 (0)	26 (8)	15 (6)
Nocardia sp. RMS6	*B. subtilis*	12 (4)	14 (7)	15 (0)	30 (8)	18 (5)
	K. pneumoniae	15 (5)	16 (3)	12 (0)	26 (8)	20 (6)

(Control values were presented in parentheses)

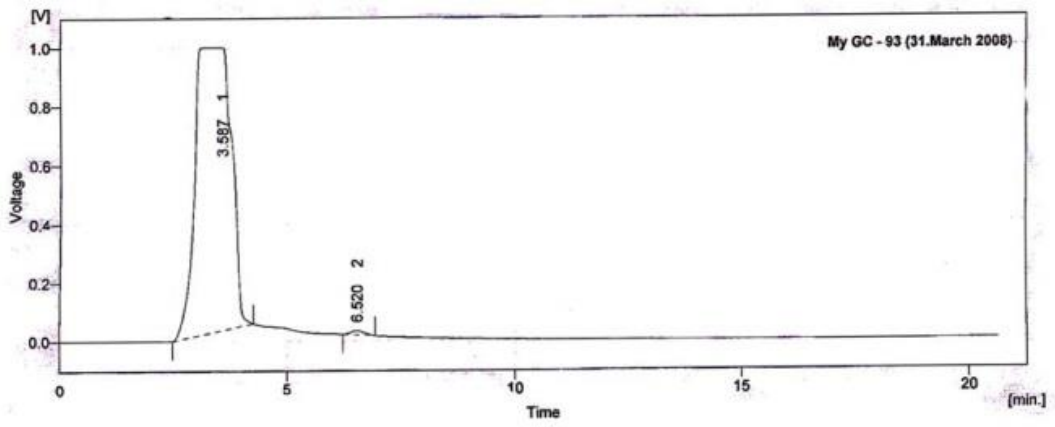

Fig.e 1. HPLC chromatogram of antimicrobial compound RMS3

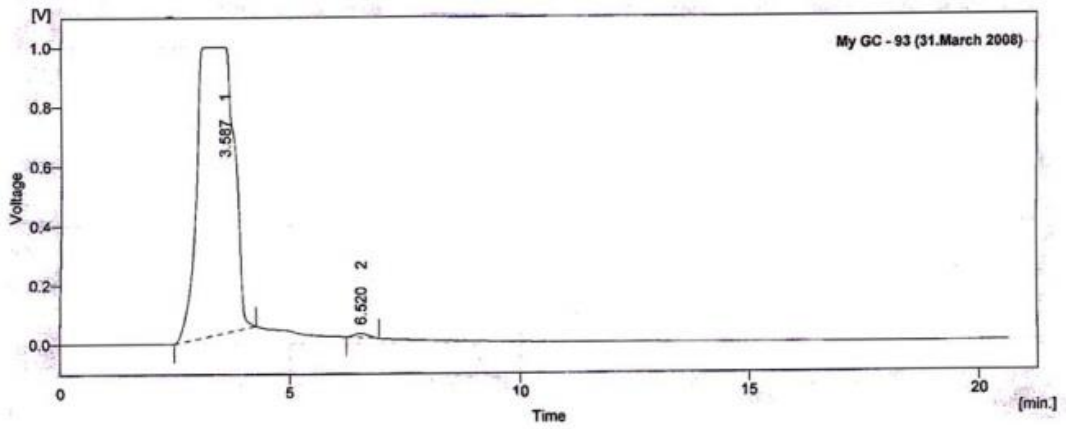

Fig. 2. HPLC chromatogram of antimicrobial compound RMS3

Table 2. Summary of chromatogram results on retention time

Name of the compound	No. of Major compound	No. of double bond	Carboxyl / hydroxyl groups	No. of methyl group	Carbon ring	Retention time
Cephalexin	One	6	2/-	-	13	3.3
Spiramycin	One	4	11 / 3	11	16	3.6

- Absence

Table 3. Cultural characteristics of the potential producers

S. No.	Name of the medium	*Streptomyces* sp. RMS3	*Nocardia* sp. RMS6
Starch nitrate agar			
	Aerial mycelium	White	White
	Substrate mycelium	Dark yellow	Light yellow
	Pigmentation	Nil	Nil
Nutrient agar			
	Aerial mycelium	White	White
	Substrate mycelium	White	White
	Pigmentation	Nil	Nil
Yeast extract malt extract (ISP 2)			
	Aerial mycelium	White	White
	Substrate mycelium	Dark yellow	Dark yellow
	Pigmentation	Nil	Nil
Oat meal agar (ISP 3)			
	Aerial mycelium	White	White
	Substrate mycelium	Dark yellow	Dark yellow
	Pigmentation	Nil	Nil
Inorganic salt agar (ISP 4)			
	Aerial mycelium	Yellowish White	White
	Substrate mycelium	Yellowish White	White
	Pigmentation	Nil	Nil
Glycerol asparagines agar (ISP 5)			
	Aerial mycelium	White	White
	Substrate mycelium	White	White
	Pigmentation	Nil	Nil

rial compound (Fig. 2). Cephalexin contains 6 double bonds, two carboxyl groups and 13 carbon rings at retention time 3.3 min. whereas, spiramycin contains 4 double bonds, 11 carboxyl groups and 3 hydroxyl groups, and showed 11 methyl groups 16 carbon rings at retention time of 3.6 min (Table 2).

Both isolates formed aerial and substrate mycelia. The strain RMS3 produced spirally twisted spores on aerial mycelium, whereas strain RMS6 produced branched vegetative mycelium. The potent isolates RMS3 and RMS6 were cultured on different media namely starch nitrate agar, nutrient agar, yeast extract malt extract agar (ISP2), oat meal agar (ISP3), inorganic salt agar (ISP4), and glycerol asparagine agar (ISP5). After incubation, white colour series of aerial and yellow colour series of substrate mycelium was produced by both RMS3 and RMS6 and no diffusible pigment was produced. Based on the colony morphology, microscopic structure, cultured, biochemical, physiological and chemotaxonomic properties of the potent two antagonistic actinobacterial isolates were identified to generic level as *Streptomyces* sp. RMS3 and *Nocordia* sp. RMS6. The cultural and other phenotypic properties like biochemical, chemo-taxonomical (whole cell sugars and cell wall amino acids) physiological (carbon and nitrogen source utilization, effect of pH, temperature and NaCl) on the growth of potential producers were recorded in Tables 3 and 4.

Discussion

Actinobacteria have been routinely screened for their high industrial value novel bioactive metabolites. These searches have been remarkably successful, approximately two thirds of naturally occurring antibiotics, including many of medical importance, have been isolated from actinobacteria [15]. In the present study, 31 morphologically distinct isolates with white and yellow coloured aerial mycelia, and yellow, white and dark yellow coloured substrate mycelia were isolated. Among

Table 4. Phenotypic properties of the potential producers

Properties	*Streptomyces* sp. RMS3	*Nocardia* sp. RMS6
Biochemical properties		
H_2S production	+	-
Nitrate reduction	-	+
Urease	-	+
TSI	Alkaline slant/ alkaline bud	Alkaline slant alkaline bud
Gelatin hydrolysis	+	-
Catalase	+	+
Oxidase	-	-
Starch hydrolysis	+	-
Casein hydrolysis	+	+
Haemolysis	+	+
Lipid hydrolysis	+	-
Melanin production	-	-
Whole cell sugar	-	-
Cell wall amino acid	-	+
Carbon source utilization		
Starch	+++	+++
Dextrose	++	-
Fructose	+	-
Maltose	++	+
Mannitol	+++	+++
Nitrogen source utilization		
L -arginine	+++	+
L -aspargine	+++	+++
L -cystine	+++	+++
L -histidine	+++	-
L -tyrosine	+++	+++
Effect of temperature (°)		
4	+++	+++
18	+++	+++
28	++++	++++
38	++	++
48	+	++
pH		
5	++	-
6	+++	+
7	+++	+++
8	+++	+++
9	+++	+++
Effect of NaCl		
Without NaCl	+++	+++
1%	++	++
2%	+	+
3%	-	-
4%	-	-

31 isolates, *Streptomyces* was the most predominant (68%) and frequently occurred in soil when compared to other genera. The dominance of *Streptomyces* among the actinobacteria especially in various soils has also been reported by many workers [16–19].

Among 31 isolates, 54.84% isolates showed antibacterial activity against Gram positive bacteria, whereas seven isolates only inhibited Gram negative bacterial growth, and 7 isolates had both Gram positive and Gram negative bacteria. From these, only 2 isolates with maximum antibacterial activity were selected for further work. The solubility of the antibacterial compounds from both the isolates were observed mostly in ethyl acetate solvent, whereas in other solvents they showed moderate to minimum solubility. It was evidenced by the antibacterial activities. In the same way, Vijayakumar et al. [20,21] tested the antimicrobial activity pattern of the marine actinobacteria against various human pathogenic bacteria and fungi, and reported that the inhibitory effect was varied depends on the pathogenic microorganisms.

High pressure liquid chromatography is being routinely used for the analytical estimation of various antibiotics [18]. In the present study, the antibacterial compounds were analyzed by HPLC analysis. Based on the retention time and absorption peaks, the antibacterial compounds were compared with the standard antimicrobial compounds [9,10,19–21]. Retention time of RMS3 compound was found similar to cephalexin, when compared with the HPLC pattern of standard antibacterial compound. Similarly, retention time of RMS6 compound was found to be spiramycin, when compared with the HPLC pattern of standard antibacterial compound. Correspondingly, similar type of HPLC analysis has been reported by Sethi [10]. For the identification of actinobacteria, ISP provided essential basic tools. In the present study, based on the colony morphology, microscopic structure, cultural, biochemical, physiological and chemotaxonomic properties of the potent two antagonistic actinobacterial isolates was identified to generic level as *Streptomyces* sp. RMS3 and *Nocordia* sp. RMS6.

Conclusion

Many of the antimicrobial drugs could not express their efficiency on all the pathogens in a same manner. Because the complexity of the cell wall of the microorganisms was different, they would protect the microorganisms from the antimicrobial drugs. Further, the productions of antimicrobial compounds have often been influenced by the components of medium, culture conditions, pH, temperature, time course etc. Extraction and purification of the compounds are essential processes for the characterization of antimicrobial compounds. Further investigation is needed in order to determine the structure of active compound and to scale up the production of metabolites.

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