Study and categorization of MDR production potential of isolated microbial *species* isolated from different areas of Rewa district

Nikita Mishra[1], Dr. Amit Tiwari[2]
[1]*Research Scholar, Centre for Biotechnology Studies, A.P.S.U, Rewa (M.P.) 486003*
[2]*Professor, Dept. of Biotechnology, Govt. T.R.S. College, Rewa (M.P.) 486001*

Abstract: During the past decades, the microbial world have provided many important bioactive compounds of highcommercial value. Over 5000 antibiotics have been identified from the cultures of Gram-positive, Gram-negative and filamentous fungi, but only about 100 antibiotics alone have been used commercially to treat human, animal,and plant diseases. These searches have been remarkably successful, and approximately two-thirds of naturally occurring antibiotics have been isolated from microbes. The need for less toxic, more potent antibiotics from noninfective organisms. Microbes, which are prolific producers of antibiotics and important suppliers to the pharmaceutical industry, can produce a wide variety of secondary metabolites. Consequently, they are continuing to be routinely screened for new bioactive substances.

Keywords: pharmaceutical industry, non-infective, Gram-positive, antibiotics.

INTRODUCTION

Soil in particular is an intensively exploited ecological niche, the inhabitants of which produce many useful biologically active natural products, including clinically important antibiotics. However, the emergence ofdrug and multidrug-resistant pathogens. The development of new antimicrobial agents, preferably naturally occurring ones with novel mechanisms of action, isan urgent medical need.[1] Searching for previously un-known microbial strains is an effective approach for obtaining new biologically active substances [2]. There is anurgent need to find new drugs, especially antibiotics, to control the spread of antibiotic resistant pathogens [3, 5] and to treat life-threatening diseases such as cancer [6]. Microbial metabolites are rich sources for new potential therapeutic drugs [7]. Over 5000 antibiotics have been identified from the cultures of Gram-positive, Gram-negative and filamentous fungi, but only about 100 antibiotics alone have been used commercially to treat human, animal,and plant diseases. The need for less toxic, more potent antibiotics from noninfective organisms, which overcome the resistance exhibited against the existing antibiotics, is felt acutely [8]. Consequently, they are continuing to be routinely screened for new bioactive substances. These searches have been remarkably successful, and approximately two-thirds of naturally occurring antibiotics have been isolated from microbes [9,10]. Microbes, which are prolific producers of antibiotics and important suppliers to the pharmaceutical industry, can produce a widevariety of secondary metabolites. Microbes are widely distributed in nature, and are typically useful in the pharmaceutical industry for their seemingly unlimited capacity to produce secondary metabolites with diverse chemical structures and biological activities [11]. Althoughthousands of antibiotics have been isolated from *Strepto- myces*, these represent only a small fraction of the repertoire of bioactive compounds produced [13,14]. Therefore,isolation of new *Streptomyces* from natural resources and characterization of their secondary metabolites is a valuable endeavor. *Streptomyces* species are Gram-positive, aerobicmicroorganisms with high DNA G C contents and produceabout half of all known antibiotics from microorganisms [15]. The species belonging to the genus *Streptomyces* constitutes 50% of the total population of soil microbes and 75 to 80% of the commercially and medicinally useful antibiotics [16].

METHODOLOGY:

Media, standard antibiotics and all other chemicals were purchased from Himedia, Mumbai, India. Freshly prepared double distilled water was used throughout the experimental work.

ISOLATION OF MICROBE FROM SOIL SAMPLES

Soil samples were collected from different hospital areas near to Rewa District. The samples were collected from 5-25 cm depth in sterile plastic bags and transported aseptically to the laboratory. The soil samples were air- dried for 1 week at room temperature. Isolation and enumeration of microbial communities were performed by serial dilution and spread plate technique. The plates were incubated at 30°C for 10 days. In our study a total of 45 microbes were isolated and designated as SCA. To minimize the fungal and bacterial growth, actidione 20 mg/L, and nalidixic acid 100 mg/L were added. One gram of soil was suspended in 9 mL of sterile double distilled water. Thedilution was carried out up to 10^{-5} dilutions. Aliquots(0.1 mL) of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were spread on the Starch Casein Agar (SCA, Himedia). [17]

MORPHOLOGICAL, PHYSIOLOGICAL, AND BIOCHEMICALOBSERVATIONS

Cultural and morphological features of SCA 7 were characterized following the methods [18]. Cultural characteristics of pure isolates in various media including, Microbes isolation agar (AIA), Mueller Hinton Agar (MHA), Starch Casein Agar (SCA), Sabouraud dextrose agar (SDA), Streptomyces agar (STP), yeast peptone glucose (YPG) agar, Zobell marine agar (ZMA), and ISP-2 were recorded after incubation at 30◦C for 7 to 14 days. The shape of cell, Gram-stain, color determination, presence of spores, and colony morphology were assessed on solid ISP agar medium. Biochemical reactions, different temperatures, NaCl concentration, pH level, pigment production, and acid or gas production were done following standard methods. Morphology of spore bearing hyphae with entire spore chains was observed with a light microscope. [19,20].

DETERMINATION OF ANTIBIOTIC SENSITIVITY ANDRESISTANCE PATTERN

Antibiotic sensitivity and resistance of *Streptomyces sp*. were assayed by the disc diffusion method [21]. A *Streptomyces* inoculums was prepared by growing cells in SCA for 48 hours at 30◦C. Petri plates were prepared with 25 mL of sterile SCA medium. The test culture was swabbedon the top of the solidified media and allowed to dry for 10 minutes. Different antibiotic loaded discs were placed on the surface of the medium and left for 30 minutes at room temperature for diffusion of the antibiotics. The plates were incubated for 48 hours at 30◦C. After incubation, the organisms were classified as sensitive or resistantto an antibiotic according to the diameter of inhibition zone given in a standard antibiotic disc chart.

CROSS STREAK METHOD

The antimicrobial activity of microbes isolates was performed by using the cross streak method [22]. YPG plateswere prepared and inoculated with isolates by a single streak in the center of the Petri plate and incubated at 30^{0}C for 7 days. The plates were then inoculated with the test organisms by a single streak at 90◦ angles to the actinomycetes strains and incubated at 37◦C overnight. Antagonism was observed by the inhibition of the test organism. The results are presented in the following manner: good activity (100% activity); moderate activity (50% activity); þ weak activity (25% activity) and no activity.

MICROBIAL ORGANISMS

The following Gram-positive and Gram-negative bacteria and some fungi were used for the experiment. Gram- positive: *Staphylococcus aureus* MTCC 96, *Micrococcus luteus* MTCC 106, *S. epidermidis* MTCC 3615, *S. aureus* ATCC 25923, and methicillin-resistant *S. aureus* (MRSA). Gram-negative: *Klebsiella pneumoniae* MTCC 109, *Enterobacter aerogenes* MTCC 111, *Salmonella typhimurium* MTCC 1251, *Shigella flexneri* MTCC 1457, *Proteus vulgaris* MTCC 1771, *Salmonella typhi-B*, and *S. typhi*. Fungi: *Candida parapsilosis* and *Malassezia pachydermatis*. The reference bacterial cultures were obtained from the Insti- tute of Microbial Technology, Chandigarh, India-160 036 andall the fungal cultures were obtained from the Departmentof Microbiology, Christian Medical College, Vellore, Tamil Nadu, India. Bacterial and fungal inocula were prepared by growing cells in Mueller Hinton broth (MHB) (Himedia) for 24 hours at 37◦C.

OPTIMIZATION OF MEDIA, MASS PRODUCTION AND INTRACELLULAR EXTRACTION

Streptomyces sp. was grown on the following media for the production of bioactive compounds in an orbital shaker (150 rpm at laboratory temperature 30°C). Antibiotic production medium, fermentation medium, glucose yeast extract malt medium, M3 medium, modified nutrientglucose (MNGA) medium, M6 medium, and YPG medium were used. The pH of the medium was adjusted to 7.0 using1M HCl and 1M NaOH. The culture was grown with continuous shaking on a rotary shaker (150 rpm) at 30°C for 14 days. After the fermentation of the culture, biomass washarvested by centrifugation (5000 rpm) at 20°C for 20 minutes, and then the mycelia were washed three times with sterile distilled water under aseptic conditions. The cells were then resuspended in a little amount of methanol and ground with the help of a pestle and mortar. Methanol wasadded to the ground cells in the ratio of 1:1 (w/v) and the mixture was shaken vigorously overnight; the extracts were then filtered through a blotting paper. The filtrates were then evaporated using a rotary evaporator at 50°C. The concentrated extract was then transferred into glass screwcap tubes and stored at 4°C for further use.

ANTIMICROBIAL ASSAY

The antibacterial activity of the crude extract was assayedusing the standard Kirby-Bauer disc diffusion method. Petri plates were prepared with 20 mL of sterile MHA (Himedia). The test cultures were swabbed on the top of the solidifiedmedia and allowed to dry for 10 minutes. The tests were conducted at 5.0 mg/disc concentrations of each crude extract. The loaded discs were placed on the surface of themedium and left for 30 minutes at room temperature for compound diffusion. A negative control was prepared using the respective solvent [dimethylsulfoxide (DMSO)]. Strep- tomycin (10 mg/disc) for bacteria and ketoconazole (30 mg/ disc) for fungi were used as positive controls. The plates were incubated over night at 37°C and zones of inhibition were recorded. Diameters of the zones of inhibition were measured using a zone scale from Himedia and expressed in millimeters. All of the experiments were done in triplicate.

FRACTIONS OF METHANOL EXTRACT AND THEIRANTIMICROBIAL ACTIVITY

The *Streptomyces sp.* was cultured, isolated, identified, and extracted for secondary metabolites according to the procedure described earlier [23]. The crude methanol extract was chromatographed over a silica gel chromatography col-umn (60e120 mesh Ramken Si gel) and eluted with mixtures of $CHCl_3$:MeOH (chloroform:methanol). Finally, 20 fractions were obtained and all were screened against microbes.

IN VITRO ANTIMICROBIAL ASSAY

× Based on biological activity, fraction 10 (8 mg) was dis- solved in 0.2 mL of DMSO and used for the antimicrobial study using the standard broth microdilution method [24]. MHB was prepared and sterilized by autoclaving at 121°C, 15 lbs for 15 minutes. The required concentrations of the extract (1000 mg/mL, 500 mg/mL, 250 mg/mL, 125 mg/mL, 62.5 mg/mL, 31.25 mg/mL, and 15.625 mg/mL) were addedto the 96 well microtiter plate containing 0.1 mL broth. Anamount of 3 mL of log phase culture was introduced into the respective well and the final inoculum size was $1\text{-}10^5$ cfu/mL. The plates were incubated at 37°C for 18 hours. Negative and solvent controls (DMSO) were also included. Streptomycin for bacteria and fluconazole for fungi were included as positive controls. An amount of 5 mLof the test broth was introduced on plain MHA plates to observe the viability of the organism. Minimum inhibitory concentration (MIC) was determined as the complete growth inhibition at a low concentration of the extract. Allthe experiments were done in triplicate.

RESULTS AND DISCUSSION

Table 1 : Preliminary screening of microbes isolates using cross streak method.

	GRAM POSITIVE					GRAM NEGATIVE			
	96	106	3615	25923		spb	spt	cp	Mp
SCA 1	-	-	+++	++		+++	++	---	++
SCA 2	+++	--	++	++		+++	--	+++	--
SCA 3	+++	++	---	++		--	+++	++	++
SCA 4	+++	--	+++	--		--	++	++	++

SCA 5	--	+++	++	++		-	-	+++	++
SCA 6	--	++	++	++		+++	--	++	++
SCA 7	++	---	++	++		+++	++	---	++

Table No. 2 : Morphological features of *Streptomyces* sp. on different media.

Media	Aerial Mycelium	Substrate Mycelium	Soluble Pigment	Reverse Side	Growth
AIA	Whitish Gray	Gray	-	Whitish	+++
SDA	-	Yellow	-	Slimy Yellow	+
STP	Dark Gray	Dark Gray	-	Yellow	+++
ISP-2	White	White	-	Whitish Yellow	++
				Yellowish Gray	+++
				Yellow	+++
				Whitish Yellow	+

- = Absent; + = Weak Growth;++ = Moderate Growth; +++ = Good Growth; Aia= Actiniomycetes Isolation Agar; ISP-2 International Streptomyes Project 2 Medium; MHA= Mueller Hinton Agar; SCA= Starch Casein Agar; SDA= Sabouraud Dextrose Agar; STA= Streptomyces Agar.

ISOLATION OF UNKNOWN MICROBIAL SPECIES

Microbes have been intensively studied in several underexplored environments, niche and extreme habitats in various parts of the world in the past few years. The soilsamples were collected from different parts of Vengodu with a view to isolate microbes strains. Based on the colony morphology and stability in subculturing, 37 suspected microbes cultures were purified on ISP-2 slants. Among 37 isolates, 13, 9, 7, 3, 3, and 2 were gray, white, green, blue, orange, and black pigmented, respectively. Interestingly, gray and white mycelial pigmented microbes were prominent in the soil. For long time storage, the strains were grown in ISP-2 broth for 7 days; then glycerol was added to make the final concentration 15% and storage was at —20°C [25]

MORPHOLOGICAL, PHYSIOLOGICAL, AND BIOCHEMICAL CHARACTERISTICS OF *STREPTOMYCES* SP.

Cultural characteristics of SCA 7 were observed after 7e14 days of growth, on eight different types of media. Strain SCA 7 grew well on AIA, SCA, STP, YPG agar, and ISP-2. Moderate growth was observed on SDA medium and weak growth was observed in MHA. The colors of col- onies were whitish gray or grayish white and grayish brown and spore chains were whitish gray in color. The spores grew better on AIA, SCA, STP, YPG agar, ISP-2, and SDA than on MHA. The pigment did not diffuse into the surrounding me- dium on any medium used. The isolate was found to be a Streptomyces sp. based on the above mentioned cultural, morphological, and biochemical characteristics. The strain SCA 7 exhibited relatively good growth between the temperatures of 25◦C and 30◦C; the optimal temperature was 30◦C. The SCA 7 isolate had tolerance limits of 11% to NaCl; test for H2S production and diffusible pigments showed negative results.

ANTIBIOTIC SENSITIVITY AND RESISTANCE PATTERN

An antibiotic sensitivity test was conducted against the mostcommonly used antibiotics for bacterial infections by meansof the disc diffusion method. SCA 7 exhibited high sensitivity towards amikacin, ampicillin, ciprofloxacin, erythromycin, gentamicin, imipenem, norfloxacin, tetracycline, and van- comycin. However, SCA 7 isolate showed resistance towards chloramphenicol, cephalothin, cephoxitin, co-trimoxazole, oxacillin, penicillin-G, piperacillin, and rifamycin.

MEDIA OPTIMIZATION

The antimicrobial activity of an organism is generally influenced by the nature of the habitat and differences in the composition of the substrate. In addition, there can bevariations in terms of different strains and the test organ- isms. For example, we found that the isolate *Streptomyces sp.* was comparatively more active than the other antagonistic isolates with a higher

antibacterial activity against Gram-positive and Gram-negative bacteria and fungal pathogens. This type of variation has also been re- ported with reference to activity against microbial pathogens in a study performed by Thumar et al., (2010) [26]. These authors isolated halotolerant alkaliphilic Streptomyces strains from the saline desert of Kutch, Western India, and determined the preliminary antimicrobial activ- ity of these isolates by the cross streak and well-diffusion methods. One of the isolates exhibited potential activity against Bacillus subtilis. Preliminary screening revealed that MNGA medium was a very good base for the production of antimicrobial compounds among the tested media. The diameters of inhibition zones produced by intracellular extracts from of SCA 7 are as follows: M. luteus (26 mm), E. aerogenes (23 mm), S. epidermidis (22 mm), S. flexneri (21 mm), P. vulgaris (20 mm), MRSA (15 mm), S. typhimu- rium (13 mm), K. pneumoniae (13 mm), S. typhi-B (10 mm),C. parapsilosis (12), and M. pachydermatis (15 mm) (Table 5).

CONCLUSION

The strain *Streptomyces sp.* isolated from an agricultural field showed a broad range of antimicrobial activiity. The methanol extract and fractions of *Streptomyces sp.* were examined for antimicrobial activity. Results showed that the methanolic fractions possessed activity; 25% showed good activity, 50% showed moderate activity, 20% showed weak activity, and 5% showed no activity against the tested microbes. More specifically, fraction 10 exhibited the best antimicrobial activity; this was sub- jected to GC-MS analysis. It showed compound 2,4-bis(1,1- dimethylethyl) phenol to be the active principle. The otherconstituents identified by the GC-MS analysis were inactivestraight chain hydrocarbons and alcohols.

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