IDENTIFICATION OF ANTAGONISTIC *STREPTOMYCES* SPECIES ISOLATED FROM WESTERN RAJASTHAN.

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ABSTRACT

Actinomycetes produce large variety of secondary metabolites of therapeutic nature. Present study was carried out to isolate Actinomycetes of antagonistic nature. In the present study soil samples were collected from different areas of Western Rajasthan. 20 isolates of Actinomycetes were isolated on Actinomycetes Isolation Agar (AIA). Several biochemical and physiological tests were performed for identification of these isolates and were screened for antagonistic potentiality against gram-positive and gram negative human pathogenic bacteria. Isolates were cultivated in fermentation media for 7 days to extract out secondary metabolites or antibiotics of antimicrobial nature. Solvent extraction method was used for purifying antimicrobial compounds from filtrate. *Staphylococcus aureus, Bacillus subtilis,Klebsiella pneumonia, Salmonella typhi* and *Pseudomonas aeruginosa* were used as test pathogen. Only isolate no. 1 and 12were found to be effective against most of the pathogens. Maximum activity of isolate no. 1 was recorded against *Klebsiella pneumonia* and minimum activity against *Pseudomonas aeruginosa* whereasisolate no. 12 showed maximum antagonism against *Staphylococcus aureus* and minimum against *Bacillus subtilis.*Chloramphenicol was used as positive control. 16S rRNA sequencing of isolate no.1 and 12 had been done and isolate no. 1 shows 99% similarity with *Streptomyces rubrolevendualae*and isolate no. 12 shows 98% similarity with *Streptomyces colicolor*. Sequences obtained from 16S rRNA sequencing were submitted in NCBI data base. From all the results we concluded that *Streptomyces* isolates showed significant antagonism against test pathogens.

Key words - *Streptomyces* , antagonism, fermentation process.

INTRODUCTION

The Actinomycetes are a prominent group of bacteria with high G+C content and possess many important and interesting features. The G + C content in DNA of Actinomycetes ranges from 57-75 %. Theyproduce80% of all known antibiotics and other therapeutically useful compounds[1]. Actinobacteria exhibit a range of life cycles which are unique among the prokaryotes and appear to play a major role in the cycling of organic matter in the soil ecosystem. Actinomycetes hold a prominent position due to their diversity and proven ability to produce new compounds, because the discovery of novel antibiotic and non-antibiotic lead molecules through microbial secondary metabolite screening is becoming increasingly important.The genus *Streptomyces* is represented in nature by the largest numbers of species and varieties among the family Actinomycetaceae. They differ greatly in their morphology, physiology and biochemical activities, producing the majority of known antibiotics. The genus *Streptomyces* includes aerobic, Gram-positive, filamentous bacteria which produce well developed vegetative hyphae (between 0.5-2.0 µm in diameter) with branches. They form a complex substrate mycelium that aids in scavenging organic compounds from their substrates. Although the mycelium and the aerial hyphae that arise from them are non-motile, motility is achieved by dispersion of spores. Spore surfaces may be hairy, smooth, spiny or warty. *Streptomyces* are noted for their distinct "earthy" odor that results from production of a volatile metabolite, geosmin[2].Due to large geographical variations in soil type and their content in Rajasthan; it is quite likely that distribution of antibiotic or secondary metabolites producing *Streptomyces* is also variable. Hence, the main thrust of this study is to screen out the *Streptomyces* with antagonistic potentiality from Western Rajasthan.

MATERIAL AND METHOD

Collection of Sample and Isolation

Soil samples were collected from various places of western Rajasthan and different Actinomycetes were isolated by serial dilution plating on Actinomycetes Isolation Agar [3] and starch casein agar [4].Plates was then incubated at 32°C for 7 days. The colonies were purified by subculturing and pure culture was preserved on Actinomycetes Isolation Agar slants at 4°C.

Optimization of media and cultural conditions:

The isolates were inoculated on different media i.e. Starch casein agar (SCA), Actinomycetes Isolation Agar (AIA), Nutrient agar (NA), Blood agar (BA) and International *Streptomyces* project (ISP) media, to determine and identify the suitable media, optimal nutritional and cultural conditions for growth. The effects of different incubation temperatures (20°, 30°,32°, 38,40 and 45°) and NaCl concentrations(1, 2, 5, 7and 9%) [5] for the growth of isolates were also studied.

Identification and Characterization of Isolates

Isolates were Characterized and Identified on the basis of their morphological, cultural, physiological, biochemical and molecular level.

Morphology:

The Morphology of Actinomycetes isolates were examined by using cover slip culture technique[6] and structure was compared with Bergey's manual[7].

Cultural Characters:

Cultural characteristics of actinomycetes isolates were examined by using different media like Actinomycetes Isolation Agar, Starch Casein Agar, Nutrient Agar, Blood agar and ISP media [8]. Color of aerial mycelium on ISP media was observed by using color scale.[9]

Physiological tests:

Different physiological tests such as melenoid pigmentation, degradation of tyrosine, xanthine, urea, citrate and hydrolysis of soluble starch and casein [10]were used to characterize the isolates

Biochemical tests:

The biochemical tests like Gram's staining, indole formation, MR-VP, Catalase enzyme and oxidase test etc. were performed and were recorded as negative or positive[7].

Test pathogen

The antagonistic potentiality was tested against six clinical isolates of human pathogens including two Gram positive *staphylococcus aureus,Bacillus subtilis* and four Gram negative bacteria viz. *Klebsiella pneumoniae, Pseudomonas aeruginosa*and *Salmonella typhi.* These isolates were collected from the Ramdeo laboratory,of Jodhpur,Rajasthan and identified microscopically and biochemically. [7]

Inocula Preparation

Bacterial strais were inoculated into 10 ml of sterile nutrient broth, and incubated at 37 °C for 24 hours. The concentration of inocula was set to 0.5 McFarland's standards[11].

Antagonistic potentiality of actinomycetes isolates:

Antagonistic activity wasperformed by using agar well diffusion assay[12]against gram positive and negative bacterial strains. Each test was performed three times and activity was expressed as the mean diameter (mm) of clear zone produced by antibacterial compounds**.** chloramphenicol was used as positive control.

Extraction of secondary metabolites for antagonism:

Extraction of secondary metabolites for antagonism had been done by fermentation of Actinomycetes isolates and filtration was done by solvent extraction method [13] .

Fermentation process

The Actinomycetes isolates were cultured at 30°C for 120 h in a jar fermentor.1 L medium containing of maltose 4%, sodium glutamate 1.2%, K_2HPO_4 0.01%, $MgSO_4$ 0.05%, $CaCl_2$ 0.01% and $FeSO_4$ 0.005% without sodium alginate beads.

Antibacterial compound was purified from the filtrate and ethyl acetate was added in ratio of 1:1 (v/v) then shaken vigorously for 1 h to complete extraction. The ethyl acetate phase contains antibiotic substances. It was evaporated to dryness in water bath. The obtained compound thus used to determine the antagonistic activity.

Molecular characterization of isolate:

16s rRNA sequencing was done by isolating and purifying the genomic DNA of isolate. The 16S rRNA fragment was amplified using universal primers (forward) i.e. 518F (SEQ: CCAGCAGCCGCGGTAATACG) and 800R (TACCAGGGTATCTAATCC). The obtained sequence was analyzed for homology using BLAST N.

RESULTS AND DISCUSSION

A total 20 isolates were isolated from soil samples.Colonies were purified on Actinomycetes isolation agar and subjected totheirantagonistic potentiality. Isolated strains were filamentous, Gram positive, non motile and aerobic in nature, having Catalase and Oxidase activities hence belonged to genus *Streptomyces*. Out of 20 isolates only isolate no.1 and 12 showed activities against tested organisms. The molecular identification of potent antibiotic producing isolate no. 1 reveals that it showed 99% similarity with *Streptomyces rubrolevendualeae and* isolates no. 12 showed 98% similarity with *Streptomyces coelicolor*.The results of Cultural characteristics of isolate no. 1 and 12are presented in table 1; biochemical and physiological characteristics are presented in table 2 and antagonistic potentiality presented in table 3.

Table-1: The cultural characteristics of *Streptomyces* on different media:

Medium	Colony color	
	Isolate 1	Isolate 12
AIA	White	Grey
SCA	Off white	Light grey
BA	White	Grey
ISP I	White	Light grey
ISP II	White	White
ISP IV	Light grey	Colorless
ISP V	Grey	Colorless
ISP VI	Yellowish	Ash grey

Table-2: The biochemical and physiological characteristics of isolate no.1 and isolate no. 12:

TESTS	**RESULTS**	
	Iso.1	**Iso.12**
Gram's staining	+	+
Colony Pigmentation	-	-
Colony color	White	Grey
Aerial mycelium	White	Grey
Hemolysis on blood agar	+	+
Hydrolysis (%w/v)of:		
Starch	+	+
Casein	+	-
Urea	-	-
Degradation (%w/v)of:		
Xanthine	-	+
Hypoxanthine	+	-
Tyrosine	-	-
Melanin production	+	-
Soluble pigment	-	+
Enzymatic activity:		
Catalase	+	+
oxidase	+	+
H2S production	-	-
Indole formation	-	-
MR test	-	-
VP test	-	-

Temperature for growth		
Optimum	34°C	37°C
Optimum pH for growth	7.5	7.5
Conc. of NaCl(%w/v)		
1%	+	+
2%	+	+
3%	-	-
5%	-	-
Carbon source utilization		
and sugar fermentation	+	+
(1% w/v)	-	-
D-glucose	-	-
Sucrose	-	-
D-xylose	+	+
D-galactose	+	+
Maltose	-	-
L-arabinose	-	-
Lactose	-	+
Inositol	-	-
Inuluin	-	-
Raffinose	-	+
Rhamnose	+	-
Fructose	-	-
Melibiose	-	-
Sorbitol	+	-
Mannitol	-	+
Mannose	-	-
Glycerol		
Nitrogen Source (1% w/v)		
Peptone	+	+
Yeast extract	+	+
Casein	+	-
Urea	-	-
Chemical characteristics		
G+C content (mol %)	59.28%	59.64%

(+) – Positive; (-) – Negative

The potent isolates were selected for fermentation on the basis of its broad spectrum antagonistic activity and largest zone of inhibition.Maximum activity of isolate no. 1 was recorded against *Klebsiella pneumonia* with 23.0±0.58 and minimum activity against *Pseudomonas aeruginosa.*Actinorhodin is an antibiotic produced by *Streptomyces coelicolor*. Hobbs etal (1996) reported that it also produced the methelomycin which founds to be effective against Gram positive and Gram negative pathogenic bacteria.[14] In present study isolate no. 12 which is identified as *Streptomyces coelicolor*showed significant antagonism against both bacteria with maximum activity against *Staphylococcus aureus*with 26.0±0.52 and minimum against *Bacillus sublilis with 6*.9±0.58. Pandey et al. (2004) and Dong-sheng Wang et al (2016) have reported broad spectrum of antibacterial Streptomyces species.[15,16]Actinomycetes isolates having antagonism against both gram positive and gram negative bacteria were isolated from Khumbu region of Nepal.[15] Antibacterial activity of culture filtrateobtained from Streptomyces sp. No. 87 against gram positive and gram negative bacteria was reported.[17] Praveen and Jain, (2007) have isolated *Streptomyces sampsonii* GS 1322 from local garden soil and reported their antifungal secondary metabolite production.[18]Deepika andKannabiran and Jodhawat et al (2012a, 2012 b) also reported antagonistic nature of actinomycetes against Gram positive and negative bacteria.[19,20,21]

Table -3 Antagonism of *Streptomyces species:*

S.NO	Clinical Isolates	Zone of Inhibition (mm)		
		Isolate 1	Isolate 12	chloramphenicol
1.	*S. aureus*	10.0±0.57	26.0±0.52	16.0±0.57
2.	*B. subtilis*	20.0±0.52	6.9±0.58	18.0±0.57
3.	*S.typhi*	11.3±0.57	12.4±0.57	15.6±0.57
4.	*K.pneumoniae*	23.0±0.58	21.0±0.52	12.8±1.0
5.	*P.aeruginosa*	5.0±0.56	14.0±0.57	15.0±1.0

Data given are mean of three replicates± Standard error. *S. aureus= Staphylococcus aureus*, *B. subtilis=Bacillus subtilis*, *S.typhi=Salmonella typhi* , K.pneumoniae= Klebsiella pneumoniae, P.aeruginosa=, Psedomonas *aeruginosa.*

The results of the present investigations reveal that growth of all the pathogens is inhibited by chloramphenicol and all the isolates; the mode of action of chloramphenicol is known as inhibitor of protein synthesis hence this mode of action is confirmed by metabolites of Actinomycetes isolates.

Nucleotide sequence accession numbers: The nucleotide sequences of 16S rRNA isolated from the isolate no. 1 and isolate no. 12 investigated in this study have been identified as *Streptomyces rubrolavendulae* and*Streptomycescoelicolor*deposited in the NCBI Gene Bank database library under accession number JN798178 and JN798179.

CONCLUSION

Streptomyces rubrolavendulae, *Streptomyces coelicolorreported* in this study shows significant antagonistic activities against some important gram negative and positive human pathogenic bacteria. It is suggested that further studies on Actinomycetes present in the Western Rajasthan's soil could provide novel species as well as novel antibiotics. According to our best knowledge *Streptomyces rubrolavendulae* is first time reported from Western Rajasthan.

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