



ANTAGONISTIC POTENTIALITY OF SECONDARY METABOLITE PRODUCING ACTINOMYCETES ISOLATED FROM SOIL OF WESTERN RAJASTHAN

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Accepted on: 06-04-2012; Finalized on: 25-05-2012.

ABSTRACT

In 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. These isolates 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 of antimicrobial nature. Solvent extraction method was used to purifying antimicrobial compounds from filtrate. *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Citrobacter koseri* were used as test pathogen. Only isolate no. 18 was found to be effective against most of the pathogens. Maximum activity was recorded against *Staphylococcus aureus* and minimum activity against *Citrobacter koseri* where as no activity was recorded against *Escherichia coli*. From all the results we concluded that Actinomycetes isolates showed significant antagonism against test pathogens.

Keywords: Actinomycetes, antagonism, secondary metabolites, fermentation process.

INTRODUCTION

Natural products are remains to be most propitious source of antibiotics⁴. There are approximately 32,500 antibiotics reported from microbial sources (Antibase Data base). Actinomycetes are the most prolific producer of 80% of known antibiotics. Actinomycetes are prokaryotes with extremely various metabolic possibilities³. The actinomycetes are Gram positive bacteria having high G+C (>55%) content in their DNA. The name 'Actinomycetes' was derived from Greek 'aktis' (a ray) and 'mykes' (fungus) and given to these organisms from initial observation of their morphology. Actinomycetes were originally considered to be an intermediate group between bacteria and fungi but now are recognized as prokaryotic organisms. The majority of actinomycetes are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants. Actinomycetes population has been identified as one of the major group of soil population¹². The group of actinomycetes that interests us most are the *Streptomycetes* because the *Streptomyces* are especially prolific and can produce a class of biologically active secondary metabolites. *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. *Streptomyces* are noted for their distinct "earthy" odor that results from production of a volatile metabolite, geosmin.

Due to large geographical variations in soil type and their content in Rajasthan, it is quite likely that distribution of antibiotic and secondary metabolites producing actinomycetes is also variable. Hence, the main thrust of

this study is to screen out the Actinomycetes with antagonistic potential from Western Rajasthan.

MATERIALS AND METHODS

Sampling and Isolation

Soil samples were collected from various habitats and 1g of soil was treated with 0.2g CaCO_3 then dried for 1hr at 100°C in oven. 20 actinomycetes isolates were isolated by serial dilution plating on Actinomycetes Isolation Agar⁸ and Starch Casein Agar¹² 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.

Identification and Characterization of Isolates

Isolates were characterized and identified on morphological¹⁵, cultural^{2,17}, physiological¹¹, biochemical¹⁶ criteria and only the isolate which showed antagonism, identified up to molecular level.

Bacterial Culture

The antagonistic potentiality was tested against six clinical isolates of human pathogenic bacteria. It includes two Gram positive *staphylococcus aureus* and *Bacillus subtilis* and four Gram negative bacteria viz. *Escherichia Coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Citrobacter koseri*. These isolates were taken from the Ramdeo laboratory, of Jodhpur, Rajasthan and were identified microscopically and biochemically¹.

Inocula Preparation

Bacterial strains 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 standards⁷.



Screening of antimicrobial activity

Primary screening of isolates have been done by cross-streak method¹⁴ and secondary screening by solvent extraction method⁵. Antimicrobial activity was performed by using Agar well diffusion assay¹⁰ 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.

Primary screening

For primary screening, actinomycetes isolates were streaked in the middle of the nutrient agar plate as straight line and allowed to grow for 48hours at 32°C and then test pathogens were streaked perpendicular to the actinomycetes and further incubated for 24 h. at 37°C.

Secondary screening:

Secondary screening has been done by fermentation of actinomycetes isolates and filtration was done by solvent extraction method.

Fermentation process

Actinomycetes were cultured at 30°C for 120 h in a jar fermentor.1 L medium containing of maltose 4%, sodium glutamate 1.2%, K₂HPO₄ 0.01%, MgSO₄ 0.05%, CaCl₂ 0.01% and FeSO₄ 0.005% with or without sodium alginate beads.

Extraction of secondary metabolites

Antibacterial compound was purified from the filtrate by solvent extraction method. Ethyl acetate was added to the filtrate in ratio of 1:1 (v/v) and shaken vigorously for 1 h complete extraction. The ethyl acetate phase contains antibiotic substances separated from the aqueous phase. 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: CCAGCAGCCGCGTAATACG) and 800R (TACCAGGGTATCTAATCC). The obtained sequence was analyzed for homology using BLAST N.

Table 1: The cultural characteristics of isolate no. 18 on different media

Medium	AIA	SCA	BA	ISP-I	ISP-II	ISP-IV	ISP-V	ISP-VI
Colony color	Off white	white	white	Off white	colorless	Light grey	colorless	Light pinkish

AIA- Actinomycetes Isolation Agar, SCA- Starch Casein Agar, ISP-International *Streptomyces* Project, BA- Blood Agar

RESULTS AND DISCUSSION

The 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 subjected for primary screening process, only isolate no. 18 showed activity against tested organisms. The molecular identification of potent secondary metabolite and antibiotic producing isolate (no. 18) reveals that it showed 99% similarity with *Streptomyces mutabilis*. The results of Cultural characteristics of isolate no. 18 are presented in table 1; biochemical and physiological characteristics are presented in table 2 and antagonistic potentiality presented in table 3.

The optimal growth of isolate was obtained in AIA medium at temperature 32°C, but did not grow at less than 20°C and above 45°. The isolate is failed to grow at acidic pH but grows well at 7.5 pH. The growth was also determined with the carbon sources for isolate no. 18. Sucrose, D-xylose, D-galactose, Maltose, Fructose, Mannitol and Mannose were utilized by isolate as carbon sources while remaining were not utilized. The isolate grows well between 1-2% NaCl conc. but optimal growth was recorded on 2% conc. Peptone, yeast, casein and urea were used as nitrogen sources. A BLAST N search of 1390 bp 16s rRNA gene sequence of the isolate shows 99% similarity with *Streptomyces mutabilis*. It was done

with the help of Macrogen genomics Korea, and isolate no. 18 was confirmed as *Streptomyces mutabilis*. The G+C DNA content of isolate *Streptomyces mutabilis* was 59.64 mol%.

The potent isolate (iso. no. 18) was selected for fermentation on the basis of its broad spectrum antagonistic activity and largest zone of inhibition. This isolate shows maximum activity against *Staphylococcus aureus* with 26±0.52mm zone of inhibition and minimum activity against *Citrobacter koseri* with 12.3±0.57 mm zone of inhibition. No activity has been shown against *Escherichia coli*. Standard antibiotic, Chloramphenicol was taken as a positive control. Fehr et al., 1977 reported that *Streptomyces mutabilis* produced mutalomycin which is effective against Gram-positive bacteria where as Suchitra et al., 2010 demonstrated anticandidal activity of *S.mutabilis*. Cross (1981) reported that fresh water actinomycetes are also good producers of antibiotics.

Nucleotide sequence accession numbers: The nucleotide sequences of 16S rRNA isolated from the isolate no. 18 investigated in this study have been identified as *Streptomyces mutabilis JN-28* and deposited in the NCBI Gene Bank database library under accession number JN798173.



Table 2: The biochemical and physiological characteristics isolate no. 18.

TESTS	RESULTS
Gram's staining	+
Colony Pigmentation	-
Colony color	Off white
Aerial mycelium	Off white
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	-
H ₂ S production	-
Indole formation	-
MR test	-
VP test	-
Temperature for growth	
Optimum	32°C
Range	20-45°C
Optimum pH for growth	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	-
Inulin	-
Raffinose	-
Rhamnose	-
Fructose	+
Melibiose	-
Sorbitol	-
Mannitol	+
Mannose	+
Glycerol	-
Nitrogen Source (1% w/v)	
Peptone	+
Yeast extract	+
Casein	+
Urea	+
Chemical characteristics	
G+C content (mol %)	59.64%

(+) – Positive; (-) – Negative

Table 3: Antibacterial activity of *Streptomyces mutabilis*

Clinical Isolates	Zone of Inhibition (mm)	
	<i>S.mutabilis</i>	Positive Control
<i>S. aureus</i>	26.0±0.52	16.0±0.57
<i>B. subtilis</i>	20.0±0.57	18.0±0.57
<i>E.coli</i>	0.0	9.0±0.57
<i>K.pneumoniae</i>	19.0±0.58	12.8±1.0
<i>P.aeruginosa</i>	16.0±0.57	15.0±1.0
<i>C. koseri</i>	12.3±0.57	15.6±0.57

Data given are mean of three replicates± Standard error. *S. aureus*= *Staphylococcus aureus*, *B. subtilis*=*Bacillus subtilis*, *E.coli*= *Escherichia coli*, *K.pneumoniae*= *Klebsiella pneumoniae*, *P.aeruginosa*=, *Pseudomonas aeruginosa*, *C. koserii* =*Citrobacter koseri*

CONCLUSION

Streptomyces mutabilis JN-28 reported in this study shows significant antagonistic activities against some important gram negative and positive human pathogenic bacteria. To our knowledge it is the first report of *Streptomyces mutabilis* JN 28 strain isolated from Western Rajasthan, antagonistic to Gram negative 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.

Acknowledgement: The authors are grateful to Prof. Swarnjeet Kaur, Head, Department of Botany, Jai Narain Vyas University, Jodhpur for providing basic research facilities.

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