Characterization of Novel Antibacterial Actinomycetes Strain N8 from Saline Soil of Vidarbha Region

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***Abstract*—Total 147 isolates were recovered from saline soil of Vidarbha region. Out of which 87 isolates (59.18 %) showed an antibacterial activity against test bacteria by agar overlay technique. In secondary screening, out of 87 primary isolates 19 actinomycete isolates were subjected for an antibacterial activity against test bacteria by agar well diffusion method. Nearly 78.94% isolates recorded antibacterial activity study towards *S. aureus* followed by 68.42% isolates to *Bacillus subtilis*, 63.15% for *Streptococcus pyogenes*, 52.63% to *Escherichia coli* and *Proteus vulgaris*, 47.36% towards *Klebsiella pneumoniae*, 42.10% for *Pseudomonas aeruginosa* and *Salmonella typhi* and 36.84% for *Enterobacter aerogenes*. Promising actinomycetes isolate (strain N8) showed antibacterial activity against all the test bacteria and was selected for morphological, cultural, physiological biochemical and genetic characterization. The 16S rRNA amplification for phylogenetic study revealed that the isolate (N8) was highly related to *Streptomyces geysiriensis* (100 %).**

***Index Terms*—actinomycetes, antibacterial, characterization, saline soil, vidarbha region.**

I. Introduction

The demand for new antibiotics continues to grow due to the rapid spread of multi drug resistant pathogen causing life threatening diseases (Berdy, 1989). The actinomycetes are important in the field of pharmaceutical industries as well as in agriculture. Antibiotics are the best known products of actinomycete. For their virtual success against pathogenic microorganisms antibiotics can be truly referred as the 'wonder drugs' (Demain, 1999). This remarkable group of compounds forms a heterogeneous assemblage of biologically active molecules with different modes of action and structures to treat bacterial infections.

Actinomycetes were predominating in black saline soils than other type like alluvial, lateric and coastal saline (Konde, 1978). Actinomycetes have more ability to bear not only at high salt concentration but also at high pH than bacteria and fungi. In uncultivated saline soil high population of actinomycetes was observed whatever may be the degree of salinity of soil (Zaharan *et al.*, 1992). In salt affected soil, the population of actinomycetes is higher at pH 7.5 to 8.0 than other pH range (Supanekar and Patil, 1995). According to Sagare *et al*., (2000) soil of saline belt of Vidarbha region is highly alkaline possessing pH ranging between 7.9 and 9.1. Hence the present study was undertaken to isolate and screen novel antibiotic producing actinomycetes from saline belt of Vidarbha region and also characterization of promising actinomycetes isolate N8.

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II. Materials & Methods

A. Collection of Soil Samples

54 soil samples were collected from 18 villages from three district of Vidarbha region, Amravati, Akola and Buldhana at different depth (10-15 cms) in sterile polythene bags with the help sterile spatula and were transported to laboratory for further processing.

B. Isolation of Actinomycetes from Saline Soil

The collected soil samples were air dried for 24-48 hours, crushed and sieved. Then soil samples were pretreated with 1% $CaCO_3$ (w/v) under humid condition to increase the number of actinomycetes propagules in the samples (Tsao *et al.*, 1993).

The Actinomycetes were isolated by serial dilution and spread plate method from collected saline soil samples on Actinomycetes isolation agar (M490, Hi-media) supplemented with 5 gm glycerol/l and antifungal antibiotic Nystatin 50 µg/ml to avoid fungal contamination (Gurung *et al.*, 2009). The isolates showed dry, tough and leathery colonies on the isolation media and purified by streak plate method on Actinomycetes isolation agar.

C. Screening of Antibiotic Producing Actinomycetes

Bond Total 147 actinomycete isolates were first primarily screened with *Staphylococcus aureus* and *Escherichia coli* by using agar overlay technique (Singh *et al*., 2006).

Isolates showing antibacterial activity against both bacteria were subjected to secondary screening against *Staphylococcus aureus* (MTCC 7443), *Bacillus subtilis* (MTCC 441), *Streptococcus pyogenes* (MTCC 442),

Escherichia coli (MTCC 443), *Pseudomonas aeruginosa* (MTCC 424), *Proteus vulgaris* (MTCC 426), *Enterobacter aerogenes* (MTCC 7325), *Salmonella typhi* (MTCC 98), *Klebsiella pneumoniae* (MTCC 7407) by using modified agar well diffusion method. The most active isolate was selected for identification.

D. *Identification of Efficient Antibiotic Producing Actinomycete Strain N8*

Promising actinomycetes strain N8 was characterized on the basis of morphological, cultural, biochemical and physiological features. The microscopic characterization was done by cover slip culture method (Kawato and Shinolue, 1959). Mycelium structure and arrangements of conidiospores on the mycelium was observed through microscope. The observed structure was compared with Bergey's Manual of Determinative Bacteriology ninth edition and the organism was identified (Holt, 1994). Cultural characteristics (growth, colouration of aerial and substrate mycelia, diffusible pigment) were tested on different media including, Tryptone Yeast Extract Agar, Starch Casein Agar, Actinomycetes Isolation Agar and Nutrient Agar with the procedure of ISP. Gram's staining was also performed. Biochemical tests including Catalase, Oxidase, Indol, Methyl Red, Voges Proskaur, Citrate utilization test, fermentation of sugars like glucose, lactose, mannitol, dextrose, galactose, sucrose, fructose, maltose and hydrolysis of starch, gelatin, urea, lipid, casein were performed by standard protocol suggested by 'International Streptomyces Project' and 'Bergey's Manual of Systematic Bacteriology'. Physiological characterization such as, effect of pH (5-8), temperature (25-40 ℃) and salinity (2-7%) and antibiotic sensitivity test against seven different antibiotics (Hi-media, Mumbai) [Amikacin (30 mcg/disc), Ampicillin (10 mcg/disc), Chloramphenicol (10 mcg/disc), Norfloxicin (10 mcg/disc), Streptomycin (10 mcg/disc), Tetracycline (10 mcg/disc) and Co-trimoxazole (25 mcg/disc)] was work out.

E. *Genetic Characterization*

The 16S rRNA gene sequencing: Efficient actinomycetes isolate N8 was further identified by 16S rRNA gene sequence analysis from National Centre for Cell Science (NCCS) Pune. The partial sequence of the 16S rRNA gene was amplified by using PCR with universal primer, 16F 27 (5'CCAGAATTGATC MTGGCTCAG-3') and 16R 1525 (5'TTCTGCAGT CTA GAAGGA GGTGWTCCAGCC-3'). The taxonomic assignment of the sequence was performed using the Ribosomal Database Project (RDP) using SEQUENCE-MATCH (Version 2.7) to identify the most closely related database sequence. In addition, the 16S rRNA sequences was compared with the Gene bank nucleotide database using the BLASTA program and multiple alignments of approximately 700 bp sequence was done by using CLUSTAL X version 1.8.

Phylogenetic analysis: The phylogenetic tree of the sequence was drawn using the software MEGA version 4.0 by neighbor joining algorithm (Tamura *et al.*, 2007) also the minimum evolutionary method as well as UPGMA method also used for the construction of the phylogenetic tree of the actinomycetes isolate. In Bootstrap, analysis (1000 replicates) was used to test the topology of the neighbor joining method data.

III. Results and Discussion

In primary screening, out of 147 actinomycete isolates 87 isolates (59.18%) showed an activity against 2 test bacteria such as Staphylococcus aureus and Escherichia coli by agar overlay technique.Out of which 45 (51.72 %) isolates active against S. aureus while 23 (26.43%) isolates active against E. coli and 19 (21.83 %) were active against both (Fig. 1). From results it is obvious that the activities against Gram positive bacteria were more frequent than against Gram negative bacteria. This frequency of activities against Gram positive bacteria is similar to previous results reported by Basilio *et al.*, (2003) and Oskay *et al.*, (2004).

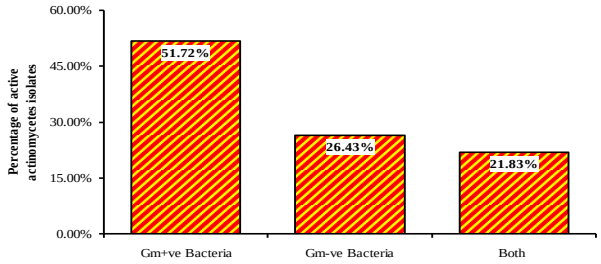

Figure 1. Primary screening of antibiotic producing actinomycetes

In secondary screening, out of 87 primary isolates 19 actinomycete isolates were preceded for an antibacterial activity against test bacteria by agar well diffusion method. Nearly 78.94% isolates recorded antibacterial activity towards S. aureus followed by 68.42% isolates to Bacillus subtilis, 63.15% for Streptococcus pyogenes, 52.63% to Escherichia coli and Proteus vulgaris, 47.36% towards Klebsiella pneumoniae, 42.10% for Pseudomonas aeruginosa and Salmonella typhi and 36.84% for Enterobacter aerogenes. Actinomycetes isolates H6, C1 and C3 showed activity against only Gram negative bacteria whereas isolates HT2, N2, N4, N5, D6, D8, C4 S6 and S9 showed activity against only Gram positive bacteria. Similarly, actinomycetes isolate N8 showed activity against all the test microorganisms. The results of secondary screening of actinomycete isolates are depicted in Table I.

The promising actinomycetes strain N8 was Gram positive, aerobic and it form circular, tough, leathery colonies that adhere to the starch casein agar surface. Cover slip culture studies indicate the spore chain morphology of N8 strain as spiral type and may place in spira group. On actinomycetes isolation agar growth was excellent with white-gray aerial mycelium and bright yellow substrate mycelium with no diffusible pigment. Again growth was excellent on starch casein agar with gray aerial mycelium and yellow substrate mycelium. On Tryptone yeast extract agar isolate showed good growth with whitish-yellow aerial mycelium and yellow

substrate mycelium. Similarly on nutrient agar good growth with creamish-white aerial mycelium and cream colour substrate mycelium was obtained. In any case diffusible pigment was not found.

TABLE I. SECONDARY SCREENING OF ACTINOMYCETE ISOLATES FOR ANTIBACTERIAL ACTIVITY BY AGAR WELL DIFFUSION METHOD.

Sr. No	Isolate code	Zone of inhibition (mm)*								
		Gram positive bacteria			Gram negative bacteria					
		S. aureus (MTCC 7443)	*B. subilis* (MTCC 441)	*S. pyogenes* (MTCC 442)	*E. coli* (MTCC 443)	*P. aeruginosa* (MTCC 424)	*P. vulgaris* (MTCC 426)	*E. aerogenes* (MTCC 7325)	*S. typhi* (MTCC 98)	*K. pneumoniae* (MTCC 7407)
1	H5	-	20±1.00	21±0.58	23±0.00	19±1.00	26±1.00	21±1.00	17±1.00	-
2	H6	-	-	-	21±1.00	-	23±0.58	-	22±1.00	19±1.00
3	HT2	21±0.00	23±1.00	-	-	-	-	-	-	-
4	KR4	19±1.00	15±1.00	19±1.00	24±0.00	21±1.00	18±1.00	17±0.58	-	22±1.00
5	N2	14±1.00	16±1.00	21±1.00	-	-	-	-	-	-
6	N3	19±1.00	21±0.00	-	24±1.00	23±0.00	25±1.00	20±1.00	18±0.58	17±1.00
7	N4	16±1.00	19±1.00	20±0.00	-	-	-	-	-	-
8	N5	19±1.00	21±1.00	22±1.00	-	-	-	-	-	-
9	**N8**	**30±0.00**	**29±1.00**	**32±0.58**	**27±0.00**	**26±0.00**	**31±1.00**	**22±1.00**	**27±0.58**	**26±1.00**
10	D1	20±1.00	-	25±1.00	26±1.00	19±1.00	16±1.00	17±0.00	24±0.00	24±0.58
11	D6	19±1.00	20±1.00	20±0.58	-	-	-	-	-	-
12	D8	21±1.00	22±1.00	-	-	-	-	-	-	-
13	Y3	20±0.58	15±1.00	23±1.00	21±1.00	-	19±1.00	17±0.00	25±1.00	18±1.00
14	C1	-	-	-	26±1.00	20±1.00	17±0.58	-	16±1.00	22±1.00
15	C3	-	-	-	21±1.00	19±1.00	24±1.00	-	22±0.58	17±1.00
16	C4	16±1.00	19±1.00	-	-	-	-	-	-	-
17	C6	25±1.00	24±0.00	17±1.00	20±0.00	26±1.00	27±0.00	19±1.00	-	21±0.58
18	S6	22±1.00	-	21±1.00	-	-	-	-	-	-
19	S9	26±1.00	-	23±1.00	-	-	-	-	-	-

*Values are mean of three replicates ± Standard Deviation (SD), (-): no zone of inhibition

The biochemical tests like Indol, Methyl Red, Voges Proskauer were negative except Citrate utilization test which was positive. Similarly, sugar fermentation was also studied and result indicates that it has capability to ferment all tested sugars such as glucose, mannitol, dextrose, galactose, sucrose, fructose with acid production except lactose which was not fermented. Also, the strain N8 produced amylase, gelatinase, lipase, protease, urease except oxidase and catalase.

The strain N8 was grown at different incubation temperatures and most favorable for growth was found to be 28 to 30 ℃. The prominent growth was obtained at pH range 6-8. Similarly, the organism showed excellent growth at 2-5% (w/v) NaCl. Isolate N8 was also subjected to the antibiotic sensitivity test by disk diffusion method. The results showed that the isolate exhibits high sensitive to Streptomycin (ZOI 27 mm) followed by Amikacin (ZOI 23 mm), Tetracycline (ZOI 21 mm), Norfloxacin (ZOI 18mm), Chloramphenicol (ZOI 17 mm). However it was found resistant to Ampicillin and Co-trimoxazole.

A. Genetic Characterization

1) Phylogenic analysis of efficient antibiotic producing actinomycete strain N8

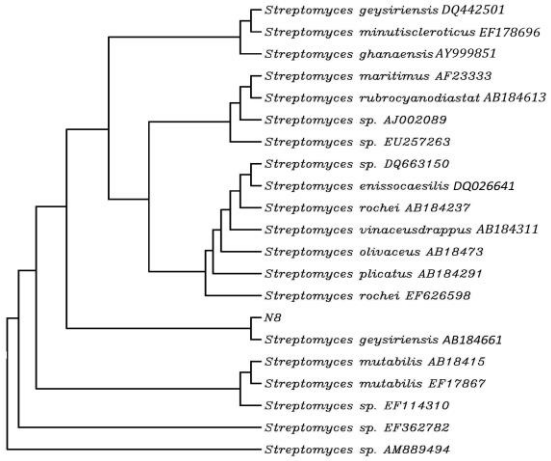

Figure 2. Phylogenetic tree based on 16S rRNA gene comparisons of the antibiotics producing actinomycete strain N8 isolated from saline soil.

The phylogenetic analysis based on 16S rRNA gene sequence indicates that strain N8 was affiliated with the phylum Actinobacteria (Fig. 2). According to the 16S rRNA gene sequence, the strain N8 showed a high level of similarity with the type strain of genus *Streptomyces* and a substantial degree of relatedness to reference 16S

rRNA sequences of genus *Streptomyces* in the database. The strain N8 from present study showed high value of similarity (100%) with isolate *Streptomyces geysiriensis* (AB184661) which was previously studied for the phylogenetic study.

Gurung *et al.,* (2009) studied antibacterial potential of seventy-nine actinomycetes from soils of Kalapatthar (5545 m), Mount Everest region. Twenty seven (34.18%) of the isolates showed an antibacterial activity against at least one test-bacteria among two Gram positive and nine Gram negative bacteria in primary screening by perpendicular streak method. Thirteen (48.15 %) showed antibacterial activity in secondary screening. Hozzein *et al.,* (2011) studied the antimicrobial activities of desert actinomycetes as potential producers of active metabolites. Out of the 75 actinomycetes strains isolated from the Egyptian desert habitats, 32 (42.67 %) showed activity against the used test organisms. Gautham *et al.,* (2011) isolated *Streptomyces* species from Western Ghats soil of Agumbe, Karnataka which were characterized on the basis of cultural, staining and biochemical tests. Kokare *et al*., (2004) reported that actinomycetes strain showed good growth in medium containing 10 to 15% (w/v) NaCl and with 30 to 36℃ temperature. The strain SU6 from 35 *Streptomyces* strains was found to be more active against five different bacterial strains. The strain was identified as *Streptomyces coelicolor* strain SU6 (JQ828940) by 16S rRNA partial gene sequencing (Usha and Selvam, 2013).

IV. Conclusions

Saline belt of Vidarbha region is a high potential source of antibiotic producing actinomycetes useful in various fields such as Pharmaceutical industries and Agricultural industries. The efficient *Streptomyces geysiriensis* (strain N8) isolated from saline soil promises to be useful in the discovery of novel antibiotic in the field of pharmaceutical industry.

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