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Original Research Article

Isolation, Identification and Characterization of Keratin degrading *Streptomyces albus*

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A B S T R A C T

In present study, 67 actinomycetes were isolated from different soils of Bellary district, Karnataka, India, by an enrichment technique. All the isolates were tested for their degradability against hair and chicken feather. Isolate VSAC-12 exhibited 92% of degradation of keratin. The typical colony of VSAC-12 was studied for morphological, biochemical and 16S rRNA gene sequence analysis which helped to identify the isolate as *Streptomyces albus*. The antagonistic activities of VSAC-12 were tested against various bacterial and fungal pathogens and its sensitivity against 16 different antibiotics was studied.

K e y w o r d s

Keratin, Degradation, Actinomycetes *Streptomyces*, 16s rRNA, Antibiotic, Antagonistic

Introduction

The actinomycetes act as natural scavengers in nature, play an important role in degrading keratinous waste and the production of keratinase. Actinomycetes are widely distributed in nature and have major role in the degradation of organic matters. They are also known as rich sources of antibiotics and bioactive molecules and are of considerable importance in industry. The ability to degrade hair and feather or other keratin-based substances, such as horn or wool, is not widely distributed among bacteria. The studied degradation of keratin and collagen containing wastes by newly iolated thermo-actinomycetes (Gousterova *et al.*, 2005). They developed a method for microbial degradation of indigenous keratin waste and to compare it with a method of alkaline hydrolysis. There are some reports on the use of keratinase and their products to degrade feather keratin more quickly and more completely (Sandali and Brandelli 2000; Ichida *et al.*, 2001; Kim *et al.*, 2001; Singh 2003). The *Streptomycetes* are aerobic, gram-positive bacteria, which produce extensive branching vegetative (substrate) mycelium and aerial mycelium bearing chains of arthro spores (Okami and Suzuki, 1958). The substrate mycelium and

spores can be pigmented, but also diffusible pigments are also produced (Williams *et al.*, 1989) on agar plates, they form lichenoid, leathery or butyrous colonies. The characterization of actinomycetes preliminarily based on color, carbon utilization (Pridham and Gottlieb, 1948), and morphology of the sporophore (Pridham *et al.*, 1958). Actinomycete taxonomy was formerly thought to be associated with morphology, which is inadequate in differentiating between different species of many genera. The use of phylogenetic and molecular evolutionary approaches has been of great importance to the classification methods (Babalola *et al.*, 2009; Hozzein and Goodfellow, 2011). Recently, the identification of the species and phylogenies are commonly derived from 16S rDNA and the use of polymerase chain reaction (PCR) for sequence analyses (Wood *et al.*, 2007; Zhi *et al.*, 2009). Since the accuracy of sequence identification is directly dependent upon the sequence database that is queried, we evaluated GenBank database (Benson *et al.*, 2004).

Soil is a natural reservoir for the microorganisms with their antimicrobial products and provides an excellent resource for the isolation and identification of therapeutically important products (Dancer, 2004). Among the soil microbes, *Streptomyces* sp. are the important group, producing antibiotics of agricultural and medicinal importance and over 6,000 compounds have been reported to be produced by *Streptomyces* (Takahashi and Omura, 2003; Kavitha *et al.*, 2010). This study aims at isolation and identification of keratin degrading actinomycetes using morphological, physiological, biochemical and 16S rRNA sequences properties. The antagonistic and antibiotic producing thermophilic actinomycetes, *Thermoactinomyces vulgaris, Streptomyces chromofuscus* and *Thermoactinomyces sacchari* isolated from compost and animal manure and identified as *Streptomyces* sp. (Makawi, 1980). They tested their antibacterial effect on the growth of some strains *of Salmonella* and *Shigella.* They found some of the *Streptomyces* had an effect on *Salmonella* but *Shigella* was not affected. Chaphalkar and Dey (1993) isolated 24 *Streptomyces* from 6 soil samples collected from Lonar lake and surrounding polluted industrial area. They tested 11 identified isolates for antagonistic activity against *Bacillus subtilis* and 8 were found to be antagonastic.

Materials and Methods

Collections of soil samples

The actinomycetes were isolated from soil samples using the method (Lacey, 1973). The soil samples were collected from different places like, compost pit area, hair and feather dumping zones and municipal wastes of Bellary district. Top layer of the soil was removed to a depth of about 8-10 cm with a clean spade and sample was collected using clean stainless steel scoop or plastic spoon. About 50 g of soil sample was collected in sterile polythene bags and the bags were labeled with date, place of collection and the sample number.

Isolation of actinomycetes

The standard serial dilution plate culture method (Nakeeb and Lechevalier, 1963) was employed to isolate the pure culture of actinomycetes. Adequate serial dilution (10^{-1}–10^{-5}) was prepared from the enriched samples. 0.1 ml of the sample from the respective dilution was plated on starch casein agar. The inoculated plates were incubated at 30^0C for one week. The growth of actinomycetes was observed on the medium at regular interval of 24 h.

Morphological characterization

A morphological study including substrate mycelium, aerial mycelium, sporulation and pigmentation status of the actinomycetes (Williams and Welington, 1980), after their adequate growth on starch casein agar was carried out as per the procedure prescribed in Bergey's manual of Systematic Bacteriology (Goodfellow, 1989). The growth characters including aerial mycelium, pigmentation and growth activity of all the 67 isolates were studied.

Determination of degradation of Hair and feather

The 67 isolates were tested for their keratin degrading ability according to Tamil Kani *et al.* (2012). 500 ml of raw hair and feather broth was prepared and autoclaved at 121°C for 15minutes. The sterile pre-weighed hair and feather pieces were aseptically transferred into respective broth. A loopful of actinomycetes cultures was inoculated into respective medium. A flask containing only the hair and feather was maintained as control. These flasks were incubated at 37°C for 10, 20, and 30 days. The percentage of degradation of hair and feather by actinomycetes was determined using the following formula

$$\text{Percentage of Weight loss} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}}$$

Electron microscopic features of VSAC-12

The ultra structure of mycelium and arrangement of spores were observed (Tresner *et al.*, 1961; Dietz and Mathews 1969) under scanning electron microscopy at the Department of Metallurgy, Indian Institute of Science, Bangalore, India.

Biochemical characterization of VSAC-12

The selected culture of VSAC-12 was subjected for Gelatin liquification, nitrate reduction, pH, temperature and utilization of carbon sources (Gottlieb, 1960).

16S rRNA gene sequencing

The culture isolated for the present research was further studied for its molecular taxonomy by sequencing and characterization of 16S rRNA gene. The 16s rRNA gene was sequenced in the laboratory of Prof. Yogesh Shouche, National Centre for Cellular Sciences, Pune University Campus, Ganeshkhind, Pune. The sequence was analyzed with the help of BLAST algorithm (Altschul Stephen *et al.*, 1997) at National Centre for Biotechnology Information (NCBI). The homologous hits of BLAST search were retrieved and further analyzed for the conserved domains using ClustalW available at European Bioinformatics Institute (EBI). The output of multiple alignment was further analyzed for the phylogenetic lineage using Phylip tool, based on neighbour joining algorithm.

Antibiotic susceptibility pattern

The antibiotic susceptibility pattern of VSAC-12 was determined by following the procedure of Collins *et al.* (1995). The antibiotics such as, Novobiocin**,** Rafampicin, Carbenicilin, Amoxycilin, Penicillin G, Rifampicin Norfloxacin Ciprofloxacin, Neomycin, Gentamicin, Acetylspiramycin, Tetracycline, Kenamycin, Streptomycin, Erythromycin and Vancomycin were used for the studies. Spore suspension of 0.1 ml containing 10^6 spores per ml was inoculated on starch casein agar by spread plate technique. After inoculation, antibiotic discs were placed carefully over the surface of agar and incubated at 30^0C for one week.

Determination of antagonistic

In vitro antagonistic activity was tested against pathogenic bacteria *viz., Escherichia coli, Bacillus substilis, Klebsiella pneumoniae, Vibro cholera, Candida albicans, Staphylococcus aureus, Pseudomonas aeruginosa,* obtained from the Department of Microbiology and Biochemistry, Gulbarga University, Gulbarga.

The antagonistic activity of VSAC-12 was determined using starch casein agar media (SCA). VSAC-12 culture was inoculated on SCA media by single streak in the center of Petri-dish and incubated at 27^0C for 4 days. Later, the plates were inoculated with test organisms. Antagonism was determined by measuring the size of inhibition zone (Madigan *et al.,* 1997; Mustafa Oskay *et al.,* 2004).

Results and Discussion

Characterization and Identification of Potent Isolate VSAC-12

Based on it's cultural, morphological, physiological and biochemical properties (Table 2). The isolate VSAC-12 was identified as *Streptomyces* sps. Further, the results of 16S rRNA sequencing and scanning electron microscopic studies (Fig. 1) revealed the isolate as *Streptomyces albus.*

Isolation and degradation of keratinolytic actinomycetes

A 67 actinomycetes were isolated from soil samples collected from different places and tested their degradability against hair and feather. Of these 57 isolates were found to be capable of using keratin substrates as a sole source of carbon and energy.

Among the isolates, VSAC-12 isolate showed a maximum of 91% and 96% degradation of hair and feather, respectively, 12 isolates showed more than 50% degradation, 23 isolates showed more than 20% degradation, 14 isolates showed a very less degradation and 10 isolates showed no degradation (Table 1). As, SGA-12 shown better degradation of keratin, was studied further.

Sequence alignment and Phylogentic Relation

Streptomyces albus was analyzed for its molecular taxonomical position by 16S rRNA gene sequencing. The sequence obtained was 1,400 bp in length (Fig. 2) and was submitted to NCBI database (Accession No EF059751). The sequence was initially characterized by BLAST analysis, which hit 100 homologous 16S rRNA genes from various species. Most homology of the sequence was found with 16S rRNA genes of *Streptomyces albus.* The homologous hits were further analyzed by ClustalW for multiple alignment, which showed the presence of conserved sequences and dendrogram was generated (Fig. 3).

Antibiotic Susceptibility Pattern of *S. albus*

The antibiotic susceptibility pattern of *S. albus* was tested against 16 antibiotics and presented in Table 4. *S. albus* was found to be resistant to Novobiocin**,** Rafampicin, Carbenicilin, Amoxycilin, Penicillin G, Rifampicin and Norfloxacin and susptibile to Ciprofloxacin, Neomycin, Gentamicin, Acetylspiramycin, Tetracycline, Kanamycin, Streptomycin, Erythromycin and Vancomycin (Table 3).

Antagonistic Activity of *S. albus*

Antagonistic activity of *S. albus* is presented in Table 5. *S. albus* showed inhibitory activity against both gram positive and gram-negative bacteria *viz., E. coli* (3.5mm), *Klebsiella pneumoniae* (3.0 mm) and *Staphylococcus aureus* (3.0 mm), *Bacillus substilis* (2.2 mm), *Candida albicans* (2.0 mm), and *Pseudomonas aeruginosa* (1.5 mm), *Vibro cholera* (1.3 mm), respectively (Table 4).

A total of 67 keratinolytic actinomycetes were isolated from the soil samples collected from different places like compost pit area, hair and feather dumping places and municipal wastes Bellary district in Karnataka, and screened them for the degradation of keratinous waste. Out of these, VSAC-12 showed 92% keratinolytic activity. The Similar findings, report these actinomycetes can be found in all kinds of soils (Kutzner, 1986; Williams *et al.*, 1983). *Streptomyces* were found (Lacey and Crook, 1988), in composts and fodder, especially in self-heated hay or grain. During the early stages of composting or self-heating, mesophilic species were present, but these were replaced by thermotolerent species like, *Streptomyces albus* or *Streptomyces griseus*, and with increasing temperature, the real thermophilic species take their place (Goodfellow and Simpson, 1987).

Streptomycetes systematic has become increasingly objective due to the application of chemotaxonomic, molecular systematic and numerical taxonomic methods (Goodfellow *et al.,* 1992; Manfio *et al.,* 1995) nevertheless, the subgeneric classification of genus *Streptomyces* in Bergey's Mannual of Systamatic Bacteriology (Williams *et al.,* 1989) is based on the extensive numerical classification generated by Williams *et al.*, (1983). The application of genetic methods such as, DNA-DNA reassociation (Labeda, 1992 and Kim *et al.*, 1999) and 16s rRNA gene sequence analysis (Gladek *et al.,* 1985; Stackebrandt and Ludwig, 1994; Kim *et al.,* 1999; Takeuchi *et al.,* 1996; Hain *et al.*, 1997) has partly confirmed the phenotypic classification, but this approach has also provided new information.

The isolate studied in the present research was identified at the molecular level by 16S rRNA gene sequencing and analysis. In the present days of molecular biology, the identification of isolates at molecular lever has become prerequisite. The sequencing of the 16S rRNA gene isolated from VSAC-12 was 1400 bp in length. The raw sequence obtained was identified with BLAST search at NCBI database.

The query sequence did hit homologous sequences, but from different species. It clearly indicates that VSAC-12 shares significant homology with the 16S rRNA genes of various microorganisms. But, the highest homology was found with *S. albus* 16S rRNA genes, which helped to recognize VSAC-12 as *S. albus.* It was interesting to note the homology of *S. albus* 16S rRNA gene with those of other species. It lead to the necessity of finding the presence of conserved domains among the hits of BLAST and it was done by multiple alignments using ClustalW tool. The results of multiple alignments clearly established the presence of various conserved domains, which spanned maximum length of the sequences analyzed. The minute variations present among the sequences analyzed were substantiated by phylogenetic analysis. The dendrogram possessed various species, forming clusters spread across a genetic distance.

Table.1 Showing percent degradation of different keratinous waste using actinomycete isolates

Sl. No.	Organisms	Human hair	Feather	Sl. No.	Organisms	Human hair	Feather
1	VSAC-1	18	21	34	VSAC-34	59	64
2	VSAC-2	22	17	35	VSAC-35	64	69
3	VSAC-3	-	-	36	VSAC-36	73	59
4	VSAC-4	48	39	37	VSAC-37	46	57
5	VSAC-5	26	39	38	VSAC-38	15	27
6	VSAC-6	19	24	39	VSCA-39	16	11
7	VSAC-7	-	-	40	VSAC-40	38	45
8	VSAC-8	-	-	41	VSAC-41	46	56
9	VSAC-9	-	-	42	VSAC-42	57	61
10	VSAC-10	09	18	43	VSAC-43	-	-
11	VSAC-11	12	24	44	VSAC-44	76	81
12	**VSAC-12**	**91**	**96**	**45**	**VSAC-45**	**59**	**44**
13	VSAC-13	16	09	46	VSAC-46	66	59
14	VSAC-14	39	51	47	VSAC-47	25	44
15	VSAC-15	27	34	48	VSAC-48	41	51
16	VSAC-16	56	48	49	VSAC-49	76	71
17	VSAC-17	18	14	50	VSAC-50	29	35
18	VSAC-18	29	21	51	VSAC-51	-	-
19	VSAC-19	57	63	52	VSAC-52	-	-
20	VSAC-20	37	50	53	VSAC-53	58	40
21	VSAC-21	49	37	54	VSAC-54	47	26
22	VSAC-22	25	29	55	VSAC-55	31	19
23	VSAC-23	09	15	56	VSAC-56	18	27
24	VSAC-24	-	-	57	VSAC-57	09	12
25	VSAC-25	11	23	58	VSAC-58	57	67
26	VSAC-26	42	58	59	VSAC-59	17	26
27	VSAC-27	59	67	60	VSAC-60	-	-
28	VSAC-28	61	59	61	VSAC-61	14	06
29	VSAC-29	18	27	62	VSAC-62	87	77
30	VSAC-30	29	24	63	VSAC-63	75	79
31	VSAC-31	35	41	64	VSAC-64	48	59
32	VSAC-32	-	-	65	VSAC-65	54	68
33	VSAC-33	-	-	66	VSAC-66	17	23
				67	VSAC-67	69	74

Table.2 Cultural and biochemical characteristic features of VSAC-12 (*S. albus* EF059751)

Test	Observation
	VSAC-12
pH	**6-10**
Temperature (^{0}C)	**25-50**
NaCl Tolerance (%) upto	**10**
Hydrolysis of:	
Casein	+
Starch	+
Cellulose	+
Pectin	–
Urea	–
Nitratereduction	–
Metanold	–
Sugar utilization	+
Raffinose	+
Galactose	+
Sucrose	+
Arbinose	+
Fructose	+
Xylose	+
Mannitol	+

+: positive –: negative

Fig.1 Scanning electron micrographs of VSAC-12

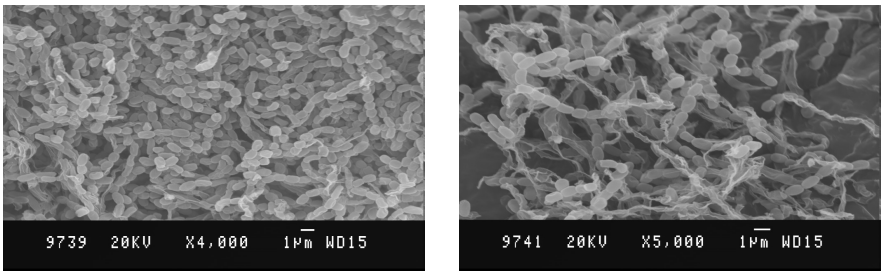

Fig.2 16S rRNA gene sequence of *S. albus* (VSAC-12)

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aacgctggcg gcgtgcttaa cacatgcaag tcgaacgatg aacccgcttc ggtggtggat
tagtggcgaa cgggtgagta acacgtgggc aatctgccct gcactctggg acaagccctg
gaaacggggt ctaataccgg atatgacacg ggatcgcatg gtctccgtgt ggaaagctcc
ggcggtgcag gatgagcccg cggcctatca gcttgttggt ggggtgatgg cctaccaagg
cgacgacggg tagccggcct gagagggcga ccggccacac tgggactgag acacggccca
gactcctacg ggaggcagca gtggggaata ttgcacaatg ggcgcaagcc tgatgcagcg
acgccgcgtg agggatgacg gccttcgggt tgtaaacctc tttcagcagg gaagaagcgc
gagtgacggt acctgcagaa gaagcaccgg ctaactacgt gccagcagcc gcggtaatac
gtagggtgcg agcgttgtcc ggaattattg ggcgtaaaga gctcgtaggc ggcttgtcgc
gtcggatgtg aaagcccggg gcttaacccc gggtctgcat tcgatacggg caggctagag
ttcggcaggg gagattggaa ttcctggtgt agcggtgaaa tgcgcagata tcaggaggaa
caccggtggc gaaggcggat ctctgggccg atactgacgc tgaggagcga aagcgtgggg
agcgaacagg attagatacc ctggtagtcc acgccgtaaa cgttgggcac taggtgtggg
cggcattcca cgtcgtccgt gccgcagcta acgcattaag tgccccgcct ggggagtacg
gccgcaaggc taaaactcaa aggaattgac gggggcccgc acaagcggcg gagcatgtgg
cttaattcga cgcaacgcga agaaccttac caaggcttga catacaccgg aaagccgtag
agatacggcc ccccttgtgg tcggtgtaca ggtggtgcat ggctgtcgtc agctcgtgtc
gtgagatgtt gggttaagtc ccgcaacgag cgcaaccctt gtcctgtgtt gccagcaact
cctttcgggg aggttgggac tcacgggaga ctgccggggt caactcggag gaaggtgggg
acgacgtcaa gtcatcatgc cccttatgtc ttgggctgca cacgtgctac aatggccggt
acaatgagct gcgatgccgt gaggtggagc gaatctcaaa aagccggtct cagttcggat
tggggtctgc aactcgaccc catgaagtcg gagtcgctag taatcgcaga tcagcattgc
tgcggtgaat acgttcccgg gccttgtaca caccgcccgt cacgtcacga aagtcggtaa
cacccgaagc cggtggccca acccttgtg ggagggagtc gtcgaaggtg ggactggcga
   ttgggacgaa gtcgtaacaa ggtagccgta ccggaa.
```

Fig.3 Dendrogram indicating the phylogenetic relation of the *Streptomycete* VSAC-12 with other organisms

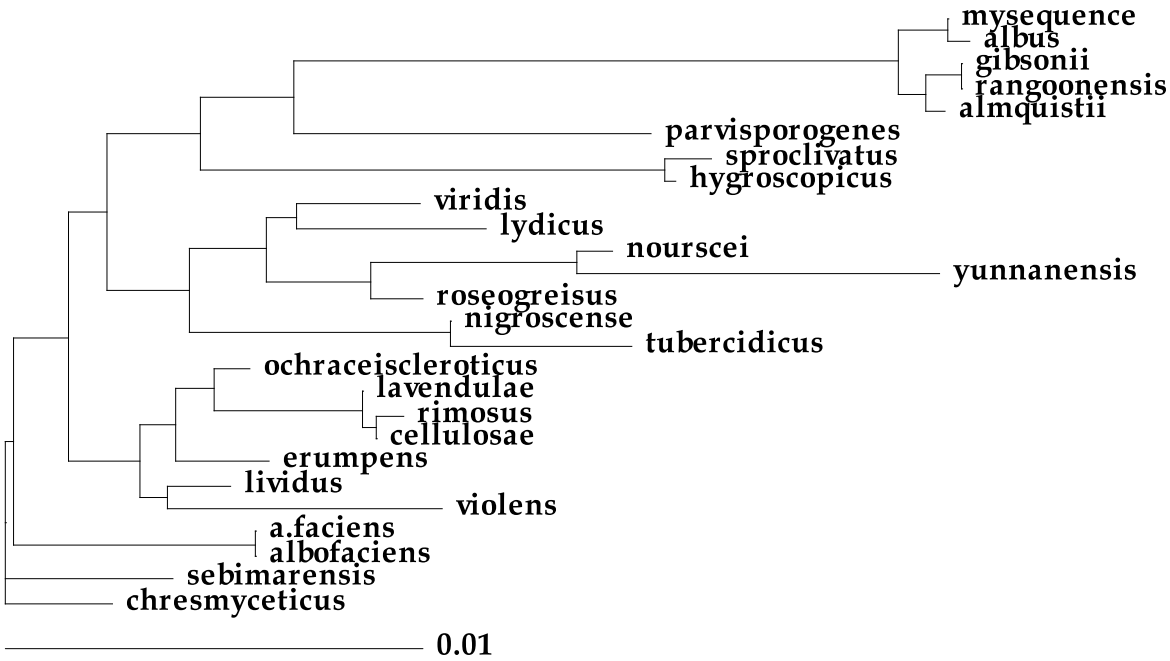

Table.3 Antibiotic susceptibility pattern of VSAC-12

Sl. No.	Test of Antibiotics	ISOLATE VSAC-12
1.	Ciprofloxacin (CIP)-5 μg/ml	S
2.	Novobiocin (NB)-30 μg/ml	R
3.	Neomycin-30 μg/ml	S
4.	Rafampicin (RA)-5 g/ml	R
5.	Carbenicilin (CB)-100 μg/ml	R
6.	Gentamicin (GM)-10 μg/ml	S
7.	Acetylspiramycin (ASP)-15 μg/ml	S
8.	Amoxycilin (AMX)-10 μg/ml	R
9.	Penicillin G (P)-10 μg/ml	R
10.	Tetracycline (TE)-10 μg/ml	S
11.	Rifampicin (RA)-5 μg/ml	R
12.	Norfloxacin (NOR)-10 μg/ml	R
13.	Kenamycin (K)-10 μg/ml	S
14.	Streptomycin-10 μg/ml	S
15.	Erythromycin (E)-15 μg/ml	S
16.	Vancomycin (VA)-30 μg/ml	S

S: Sensitive, R: Resistance,

Table.4 Antagonastic activity of *S. albus*

Sl. No.	**Test organisms**	**Zone of inhibition**
1.	*E. coli*	3.5
2.	*Bacillus subtilis*	2.2
3.	*Klebsiella pneumoniae*	3.0
4.	*Vibro cholerae*	1.3
5.	*Candida albicans*	2.0
6.	*Staphylococcus aureus*	3.0
7.	*Pseudomonas aeruginosa*	1.5

The main query sequence shared highest homology with *S. albus* retrieved from NCBI database, signifying the BLAST results.

It was interesting to note that other three species share highest similarity with *S. albus*. The major variation of query sequence was found with that of *Chresmyceticus* as these two sequences are put at highest genetic distance, as indicated by the dendrogram. So many other sequences retrieved from different species also found to have significant similarity among themselves and also with query sequence. The bioinformatics tools have helped to position the various species at the specific taxonomical level and proved handy in the present research. This study has formed the basis of the classification of the species of genus *Streptomyces* in Bergey's manual (Williams *et al.,* 1989).

In present study *Streptomyces albus* showed resistance to various antibiotics. Williams and Devis (1965) first observed actinomycetes resistance to penicillin. Ivanitskaya *et al.,* (1978) reported resistance of *Micropolyspora* to gentamycin. Isiawa and Arargi, (1976) have isolated antibiotic resistance actinomycetes. Kulkarni and Desmukh (2001) reported penicillin G resistance in 70 isolates and gentamycin in 43 isolates of 112 actinomycete isolates. The antagonistic activity against different test organisms by study *S. albus* showed inhibitory activity against both gram positive and gram negative bacteria. This trend is well known phenomenon and observed by various workers like Rangaswami *et al.,* (1967), Hamid *et al.,* (1980), Bordoloi *et al.,* (2001), Lee and Hwang (2002).

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