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ORIGINAL ARTICLE

Taxonomy and antimicrobial activity of moderately salt-tolerant and alkaliphilic *Streptomyces* sp. MN 9(V) isolated from solitary wasp mud nest

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Abstract An actinobacterium strain designated as MN 9 (V) was isolated from solitary wasp mud nest obtained from the walls of buildings located in Dehradun, India. MN 9(V) was subjected to polyphasic taxonomic characterization. The organism produced light-grey aerial mycelium with brown-coloured diffusible pigment. This isolate showed growth in medium containing 8% NaCl, at pH 8–10 and at a temperature range of 27–45°C. Micromorphology and chemotaxonomic characteristics were consistent with its assignment to the genus *Streptomyces*. Analysis of 16S rDNA gene sequences showed that MN 9(V) formed a separate clade with *Streptomyces cellulosae* NRRLB-2889^T and *Streptomyces pseudogriseolus* NRRL B-3288^T being most closely related with a sequence similarity of 99%. However, a number of phenotypic characteristics readily distinguished the strain MN 9(V) from the type strains. The strain MN 9(V) showed strong antimicrobial activity against several test organisms, including multidrug resistant bacteria, as well as against fungal pathogens.

Keywords Taxonomy · *Streptomyces* · Multidrug resistant · Solitary wasp mud nest

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Introduction

The search for new antibiotics continues to be of great importance in screening programs around the world because of the increase in resistant pathogenic bacteria and the toxicity of some currently used antibiotics (Berdy 1989). The history of new drug discovery processes showed that, in the majority of cases, novel skeletons have come from natural sources (Bevan et al. 1995). Among micro-organisms, actinomycetes are one of the most attractive sources of all types of bioactive metabolites that have important applications in human medicine (Watve et al. 2001). The metabolic diversity of the actinomycete family is due to its extremely large genome, which has hundreds of transcription factors that control gene expression, allowing them to respond to specific needs (Goshi et al. 2002). In this context, actinomycetes producing antibiotics that inhibit the growth of the ant parasite *Escovopsis* were isolated from fungus-growing ants (Cafaro and Currie 2005; Currie et al. 1999). *Streptomyces* sp. are also found frequently in pollen, and in the alimentary canals of alfalfa leafcutter bees (*Megachile rotundata*), and these bacteria are considered as part of the resident microflora of the bee (Inglis et al. 1993). Recently, mycangimycin, which appears to be responsible for the selective inhibition of an antagonistic fungus of the mutualistic fungus associated with *Dendroctonus frontalis* beetles, has been reported from *Streptomyces* sp. (Scott et al. 2008). A novel compound, dentigerumycin, was obtained from a fungus-growing ant-associated actinobacterium (genus *Pseudonocardia*), which aids in the protection from a specialized parasite of the ants' mutualistic fungus (Poulsen et al. 2010).

Recently, 200 isolates of *Streptomyces* species were isolated from two species of solitary wasps. Chemical analyses of 15 of these isolates identified 11 distinct and

structurally diverse secondary metabolites, including a novel polyunsaturated and polyoxygenated macrocyclic lactam, named as sceliphrolactam (Poulsen et al. 2011). By pairing the 15 *Streptomyces* strains against a collection of fungi and bacteria, the authors documented their antifungal and antibacterial activity. The prevalence and anti-microbial properties of Actinobacteria associated with solitary wasp species suggest the potential role of these *Streptomyces* as antibiotic-producing symbionts, potentially helping defend their wasp hosts from pathogenic microbes (Poulsen et al. 2011). On the basis of the above studies we focused on solitary wasp mud nests for the isolation of potential actinomycetes active against drug-resistant bacterial pathogens. The present report highlights the taxonomy and antimicrobial activity of a new moderate salt tolerant akaliphilic actinomycete strain isolated from solitary wasp mud nest, a rare habitat.

Materials and methods

Selective isolation of actinomycetes

Actinomycetes were isolated from solitary wasp mud nest collected from Dehradun, India using Humic acid vitamin agar medium (HV; Hayakawa and Nonomura 1987a, b; Hayakawa et al. 1996). Prior to isolation, soil samples were heat treated at 120°C for 1 h (Hayakawa et al. 1991) then 1.0 g soil from each sample was dissolved in 10 ml sterile normal saline and serially diluted up to 10^{-4} and plated (1 ml) on HV agar medium. All plates were incubated at 27°C for 15 days.

Morphological and physiological characteristics

Cultural characteristics of the isolates grown on various International *Streptomyces* project (ISP) media (Shirling and Gottlieb 1966) were examined daily. Micromorphology and sporulation were observed under light microscope using the inclined coverslip technique (Williams et al. 1989) on ISP-4 medium after incubating at 27°C for 7 days. Spore chain morphology and spore surface ornamentation was examined by scanning electron microscopy according to the method described by Kumar et al. (2011). Physiological characteristics were examined according to the methods described in the ISP (Shirling and Gottlieb 1966) and *Bergey's Manual of Systematic Bacteriology* (Locci 1989). Resistance to antibiotics was detected by the disc diffusion method.

Chemotaxonomic analysis

The isomeric forms of diaminopimelic acid (DAP) and diagnostic sugar in the whole-cell hydrolysates were determined as described in *MTCC Laboratory Manual*, IMTECH, Chandigarh, India (IMTECH 1998).

Molecular identification

The most promising isolate with desirable antimicrobial properties was identified using molecular approaches. Chromosomal DNA of the strain MN 9(V) was prepared from cells grown in nutrient broth for 2–3 days incubation according to described methods (Kumar et al. 2010). PCR amplification of the 16S rDNA of the strain MN 9(V) was performed using two primers 27F (5′-AGAGTTTGATC (AC) TGCCTCAG-3′) and 1492R (5′-GGTTACCTTGT TACGACTT-3′). The reaction mixture (final volume 25 μl) contained PCR buffer F, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 20 pmol of each primer, 2.5 U *Taq* DNA polymerase (Genei, Bangalore, India) and 100 ng template DNA. The amplification was performed in an Eppendorf Thermo-cycler 96, using the following profile: an initial denaturation step at 94°C for 2 min, followed by 30 amplification cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and a final extension step of 72°C for 2 min. The PCR product was detected by agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence after ethidium bromide staining. Prior to sequencing, amplified products were purified using a HiPurA™ PCR product purification spin kit (HiMedia Laboratories, Mumbai, India) according to the manufacturer's instructions. Sequencing reactions of PCR products were performed with the ABI PRISM® Big Dye® Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions using 9F and 1541R primers.

Phylogenetic and molecular evolutionary analyses were conducted using software included in the MEGA version 4.0 package (Tamura et al. 2007). The 16S rDNA sequences of the strain was aligned using CLUSTAL W program (Thompson et al. 1994) against corresponding nucleotide sequences of the *Streptomyces* retrieved from GenBank, EMBL, DDBJ and RDP. Evolutionary distance matrices were generated as described by Jukes and Cantor (1969) and a phylogenetic tree was inferred by the neighbor-joining method (Saitou and Nei 1987). Tree topologies were evaluated by bootstrap analysis (Felsenstein 1985) based on 1,000 resamplings of the neighbor-joining dataset.

Determination of antimicrobial activity

Spores from a mature slant culture of the strain MN 9(V) were inoculated aseptically into a 250-ml Erlenmeyer flask containing 30 ml seed medium NB (beef extract 1 g/l, yeast extract 2 g/l, peptone 5 g/l, sodium chloride 5 g/l, final pH 9.0) and incubated in a rotary shaker at 30°C for 2 days

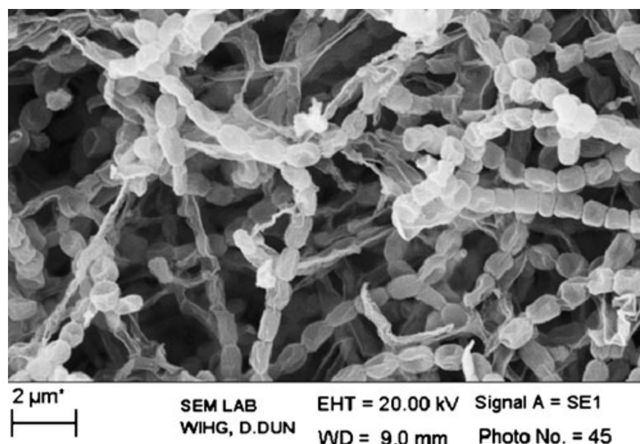

Fig. 1 Scanning electron micrograph showing spore chain morphology of *Streptomyces* sp. MN 9(V). *Bar* 2 μm

at 170 rpm. The seed culture (4 ml) was transferred into 40 ml GS medium (glucose 10 g/l, soya bean meal 10 g/l, sodium chloride 10 g/l, calcium chloride 1 g/l, pH 9) in a 250 ml flask and incubated at 30°C for 4 days under agitation at 200 rpm. After incubation, the fermented broth was centrifuged at 10,000 rpm for 5 min. and filtered through Whatmann No.1 filter paper. The culture filtrates were assayed for antimicrobial activity using the agar well diffusion method. Muller Hinton (HiMedia) agar plates were inoculated with bacterial pathogens (*Staphylococcus aureus* MTCC 2940, *S. aureus* MTCC 96, *S. aureus* clinical-1, *S. aureus* clinical-2, *S. aureus* clinical-3, *Bacillus subtilis* MTCC 441, *Escherichia coli* MTCC 739, *E. coli* clinical, *Pseudomonas aeruginosa* MTCC 424, *P. aeruginosa* clinical, *Acinetobacter baumannii* MTCC 1425, *Acinetobacter junii* MTCC 1686, *Salmonella* sp., and *Micrococcus luteus* MTCC 106) except *Mycobacterium smegmatis*, which was inoculated on Middlebrook 7H10 agar base (HiMedia), whereas Sabouraud dextrose agar (peptone 10 g /l, dextrose 20 g/l, agar 15 g/l, final pH 5.6) plates were inoculated with fungal pathogens (*Trichophyton rubrum* MTCC 296 and *Candida albicans* MTCC 1637) and wells made using a cork borer (6 mm diameter).

Table 1 Culture characteristics of *Streptomyces* sp. MN 9(V)

Medium	Growth	Aerial mycelium	Substrate mycelium
Yeast extract- malt extract agar (ISP-2)	Good	Greyish white	Brown
Oatmeal agar (ISP-3)	Good	Grey	Brown
Inorganic salt-starch agar (ISP-4)	Good	Grey	Dark brown
Glycerol asparagine agar (ISP-5)	Poor	Light grey	Colorless
Tyrosine agar (ISP-6)	Good	White	Yellow
Actinomycete isolation agar	Moderate	Brown	Colorless

Table 2 Phenotypic characteristics of *Streptomyces* sp. MN 9(V)

Characteristic	MN 9(V)	*Streptomyces cellulosae*[T b]
Spore mass	White to grey	Yellow to grey
Spore surface	Smooth	Smooth
Spore chain	Rectiflexibilis	Rectiflexibilis
Spores per chain	10–50	3–10
Starch hydrolysis	+[a]	+
Casein hydrolysis	-	+
Gelatin hydrolysis	-	+
Oxidase	-	ND
Catalase	-	ND
H_2S production	+	+
Diffusible pigment	+, Brown	Red orange
Melanin pigment	-	-
Nitrate reduction	-	-
C-Utilization		
Glucose	+	+
Rhamnose	+	V
Maltose	+	ND
Arabinose	-	+
Sucrose	-	+
Raffinose	+	-
Salicin	+	+
Mannose	+	-
N- Utilization		
L-Serine	+	-
L-Phenylalanine	+	-
L- Threonine	+	V
Methionine	+	-
Hydroxyl proline	+	V
L- Histidine	+	V
Potassium nitrate	+	ND
Growth at different NaCl conc.		
0-7%	+	+
8%	+	-
Growth at		
45°C	+	+
Antimicrobial activity		
Micrococcus luteus	+	V
Bacillus subtilis	+	V
Candida albicans	+	-
Aspergillus niger	+	ND

[a] +, Activity present; -, Activity absent; V, Variable; ND, Not determined

[b] Data from Locci 1989

Culture filtrate (50 μl) was added to each well, incubated and observed for antimicrobial activity in terms of growth inhibition zones. All assays were performed in triplicate. A

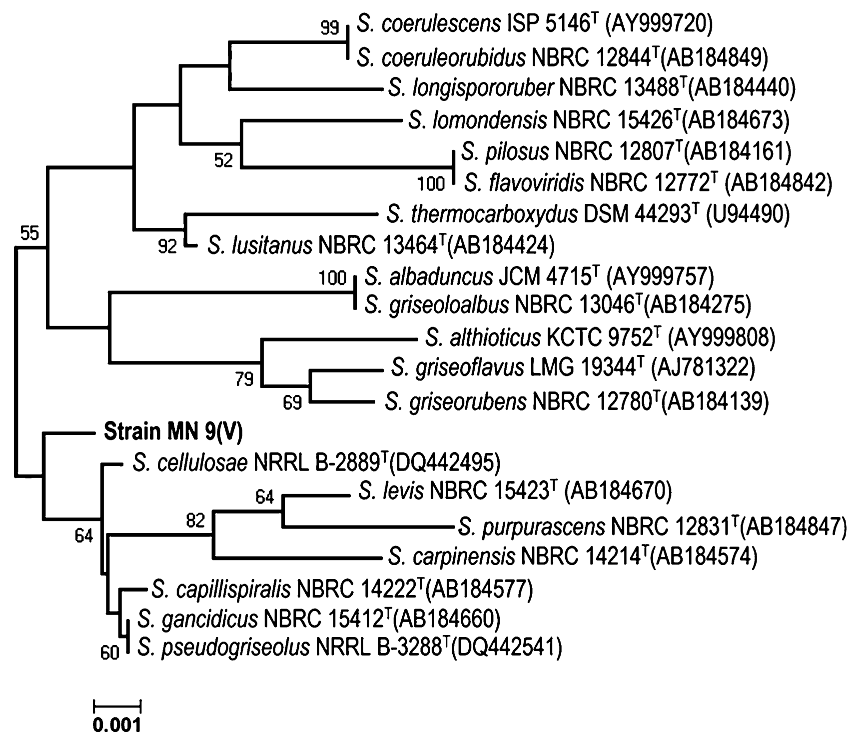

Fig. 2 Neighbor-joining phylogenetic tree based on 16S rDNA gene sequences, showing the relationships between strains MN 9(V) and the most closely related type strains of *Streptomyces*. The numbers at nodes indicate percentages of bootstrap support based on a neighbor-joining analysis of 1,000 resampled datasets; only values above 50% are given. *Bar* 0.001 substitutions per nucleotide position. *T* Type strains

negative control of uninoculated culture broth as well as a positive control of streptomycin sulphate (10 μg/well), rifampicin (5 μg/well) and vancomycin (30 μg/well) was used.

Results

A total of 65 actinomycetes were isolated from solitary wasp mud nests collected from different buildings located at Dehradun, India. A total of eight genera were recovered from solitary wasp mud nest (*Streptomyces*, *Streptosporangium*, *Saccharopolyspora*, *Nocardia*, *Nocardiopsis*, *Actinomadura*, *Thermoactinomycetes* and *Micromonospora*). The genus *Streptomyces* was dominant as compared to other genera. All the isolates were screened for their antimicrobial properties. The antimicrobial activity varied greatly among the actinomycetes isolates. Out of 65 isolates 34.09 % (n=15 showed activity against *S. aureus* MTCC 96. Out of 15 active isolates, MN 9(V) showed most prominent activity against most of the tested drug resistant bacterial pathogens, hence this strain was chosen for detailed study.

Strain MN 9(V) produced extensively branched substrate and aerial hyphae. Scanning electron microscopy of the strain revealed that aerial mycelia of strain MN 9(V) had long chains of spores (up to 45–50 spores per chain). The spore surface of MN 9(V) was smooth (Fig. 1).

Culture characteristics of *Streptomyces* sp. MN 9(V) are shown in Table 1. The strain grew well on all media except inorganic salt-starch agar, in which poor growth was observed, while growth was moderate on actinomycetes isolation agar. Aerial mycelium was initially white and gradually darkened to grey. Likewise, vegetative mycelium was brown. Diffusible pigment was produced in ISP-2 medium while no melanin pigment was produced on ISP-6 and ISP-7.

The physiological characteristics of strain MN 9(V) are given in Table 2; the strain is moderately thermotolerant, the optimum range for the isolate being between 27°C and 45°C. It can utilize all major nitrogen and carbon sources except arabinose and sucrose. It grew at a salt concentration of 8% NaCl (w/v) , pH 8–10 and was found to be resistant against Penicillin G (10 IU), Co-trimoxazole (25 μg), Erythromycin (10 μg), Lincomycin (10 μg), Azetreonam (30 μg) and Cefuraxime (30 μg).

Chemotaxonomic tests showed that whole-cell hydrolysates of strain MN 9(V) were rich in LL- diaminopimelic acid (LL-DAP), while no characteristic sugar indicated a chemotype I. Chemotaxonomic and morphological properties of the strain MN 9(V) are in line with its classification in the genus *Streptomyces* (Williams et al. 1989).

The 16S rDNA sequence of the strain MN 9(V) was determined and submitted to GenBank under the accession number HM991287. The sequence was first analyzed by BLAST search and was then aligned with those of *Streptomyces* reference strains available in the GenBank database, which confirmed its identification as strain MN 9 (V) at genus level. Its position among the type strains of *Streptomyces* is shown in Fig. 2. An almost complete 16S rDNA gene sequence for strain MN 9(V) was determined in

Table 3 Antimicrobial activity of *Streptomyces* sp. MN 9(V)

Test organism	Diameter of clear zone (mm)	Standard antibiotics[a]		
		Str	R	V
Staphylococcus aureus MTCC 2940 (resistant to Methicillin, Penicillin G and Streptomycin)	20	-	17	22
S. aureus MTCC 96	21	25	22	23
S. aureus clinical-1 (resistant to Penicillin G and Methicillin)	19	20	32	20
S. aureus clinical-2 (β- lactamase positive)	20	18	30	20
S. aureus clinical-3 (β- lactamase positive)	18	18	30	20
Bacillus subtilis MTCC 441	18	24	18	22
Escherichia coli MTCC 739	20	22	11	-
E. coli clinical (resistant to Bacitracin, Cephalothin, Co-trimoxazole, Erythromycin and Penicillin-G)	18	11	-	-
Pseudomonas aeruginosa MTCC 424	13	17	-	-
P. aeruginosa clinical (resistant to Amikacin, Ampicillin, Chlorophenicol, Cephalothin, Co-trimoxazole, Cephotaxime, Gentamicin, Nalidixic acid, Nitrofuratoin, Norfloxcin, Streptomycin and Tetracycline)	11	15	-	-
Acinetobacter baumannii MTCC 1425 (resistant to Ampicillin, Cephalothin, Cephotaxime, Bacitracin, Nitrofuratoin and Streptomycin)	22	-	13	-
Acinetobacter junii MTCC 1686 (resistant to Cephotaxime and Nitrofuratoin)	19	-	35	30
Salmonella sp. (resistant to Rifampicin)	18	17	-	-
Mycobacterium smegmatis	17	40	-	33
Micrococcus luteus MTCC 106	23	27	22	24
Trichophyton rubrum MTCC 296	20	ND[b]	ND	ND
Candida albicans MTCC 1637	18	ND	ND	ND

[a] *Str* Streptomycin sulphate (10 μg/well); *R* Rifampicin (5 μg/well); *V* Vancomycin (30 μg/well); - activity absent

[b] Not determined

this study. A phylogenetic tree was constructed based on 16S rRNA gene sequences to show the comparative relationship between strain MN 9(V) and other related *Streptopmyces* species (Fig. 2). The comparative analysis of 16S rRNA gene sequence and phylogenetic relationship showed that strain MN 9(V) lies in a separate clade in the tree with *Streptomyces cellulosae* NRRLB-2889^T and *Streptomyces pseudogriseolus* NRRL B-3288^T, with which it shares a 16S rRNA gene sequence similarity of 99%.

The antimicrobial activity of the strain MN 9(V) is given in Table 3. The strain MN 9(V) inhibited growth of Gram-positive, Gram-negative bacteria and fungi, suggesting broad spectrum characteristics of the active metabolite.

Discussion

One of the more efficient ways of discovering novel metabolites from microorganisms is through the isolation of new microbial species. In this context, solitary wasp mud nest was taken as a substrate for study. The distribution of rare actinomycetes in this study was lower than that of streptomycetes, confirming previous reports from various types of soil, and environments such as tropical rainforest, sea-mud and natural caves, Moroccan habitats, beehives, wasp-associated actinobacteria and some other less explored ecosystems like desert, forest soil and marine sediments (Mitra et al. 2008; Mukku et al. 2000; Promnuan et al. 2009; Radhakrishnan et al. 2010; Poulsen et al. 2011). However, the results were comparable with a previous study of actinomycetes in terrestrial soil from the same location (Mathpal and Bisht 2007). None of the isolates (from terrestrial soil) showed promising activity against all the tested pathogens (Mathpal and Bisht 2007), whereas from solitary wasp mud nest, out of 15 active isolates, 1 isolate, designated as MN 9(V), showed very promising antimicrobial activity and hence was chosen for further detailed study.

MN 9(V) is a moderate thermotolerant and alkaliphilic microorganism isolated from solitary mud wasp nest. MN 9 (V) was identified as belonging to the genus *Streptomyces* on the basis of morphological, physiological and biochemical characteristics, including cell wall constituents. The results of phylogenetic analysis based on almost complete 16S rDNA sequence confirmed its placement in the genus *Streptomyces*.

The strain MN 9(V) shared a 16S rDNA sequence similarity of 99% with *Streptomyces cellulosae* NRRLB-2889^T and *S. pseudogriseolus* NRRL B-3288,^{T} *S. gancidicus* NBRC 15412^T, *S. capillispiralis* NBRC 14222^T, *S. thremocarboxydus* DSM 44293^T and *S. lusitanus* NBRC 13464^T. High sequence similarities and nucleotide differences of similar values have been recorded for the type strains of many *Streptomyces* species, some of which are known to have DNA–DNA relatedness value lower than the 70% cutoff point recommended for delineation of genomic species (Wayne et al. 1987). For instance, the type strains of *S. cellulosae* NRRLB-2889^T, *S. thermocarboxydus*, *S. thermodiastaticus* and *S. thermoviolaceus* susp. *thermoviolaceus* share high 16S rDNA sequence similarity values with one another (98.7–99%) but can be separated on the basis of genotype and phenotype properties (Kim et

al. 1998, 2006; Kim and Goodfellow 2002; Manfio et al. 2003). Currently, 16S rDNA gene sequence similarity of 98.7–99% is proposed as mandatory for defining the genomic uniqueness of a novel isolate (Stackebrandt and Ebers 2006).

Although strain MN 9(V) shares high 16S rDNA similarity value with *S. cellulosae*, these two organisms differ in a number of phenotypic characteristics. Strain MN 9(V) produced brown diffusible pigment on ISP-2, and hydrolyses starch. Spore chain is rectiflexibilis, with 10–50 spores per chain. Strain MN 9(V) utilized raffinose, cellobiose, fructose, inositol, xylose, mannose but not arabinose and sucrose while arabinose and sucrose were utilized by *S. cellulosae*. All nitrogen sources tested were utilized by strain MN 9(V) while *S. cellulosae* did not utilize L-serine, L-phenlyalanine or methionine. Strain MN 9(V) was able to grow in the presence of 8% NaCl (w/v) while *S. cellulosae* grew at a salt concentration of 7% (w/v). MN 9(V) also differed in antimicrobial activity against *Candida albicans*. Therefore, strain MN 9(V) may represent a novel species of *Streptomyces*.

The antimicrobial activity of MN 9(V) was screened against various Gram-positive and Gram-negative bacteria and fungal species. MN 9(V) showed broad spectrum antimicrobial activity against various multidrug resistant Gram-positive bacteria, such as *Staphylococcus aureus*, and Gram-negative organisms, like *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter baumannii* and *Acinetobacter junii* (Table 3). Strain MN 9(V) also showed antimycobacterial as well as antifungal activity. Our results were comparable to those of Poulsen et al. (2011), who reported 200 isolates of *Streptomyces* actinobacteria associated with wasps. Out of 200 isolates, 15 *Streptomyces* strains showed activity against fungi and bacteria. Although the chemical analysis of 15 of these isolates identified 11 distinct and structurally diverse secondary metabolites, including a novel polysaturated and polyoxygenated macrocyclic lactam, named as Sceliphrolactam (Poulsen et al. 2011), there was no record of their antimicrobial activity against human bacterial and fungal pathogens. Our results were also comparable with those from *Streptomyces* species isolated from other habitats. Dhanasekaran et al. (2009) reported the antimicrobial activity of *Streptomyces* strain DPTD-5, which inhibited the growth of some pathogenic bacteria (*S. aureus*, *E. coli*, *P. aeruginosa* and *Bacillus* sp.) and fungi (*C. albicans*, *C. tropicalis*). Srivibool et al. (2010) reported the antimicrobial activity of *Streptomyces* strain CH54-4, which inhibited the growth of both Gram-positive (*B. subtilis*, *S. aureus* MRSA, *Micrococcus luteus*) and Gram-negative (*P. aeruginosa*) bacteria, yeasts (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Debaryomyces hansenii*, *C. albicans*) and fungi (*Pichia dispora*, *Mucor racemosus*, *Aspergillus niger*, *Penicillium chrysogenum*), suggesting the broad spectrum nature of the active compound(s). However, the antimicrobial pattern of *Streptomyces* sp. MN 9(V) was different from that of *Streptomyces* strain CH 54–4. Studies carried out by other workers also revealed that salt-tolerant akaliphilic actinomycetes are rich in bioactive antibiotics (Kokare et al. 2004; Manam et al. 2005; Thumar et al. 2010). A comparison of the antimicrobial pattern of actinomycete isolate MN 9(V) with that of other actinomycetes is given in Supplementary Table 1.

Nowadays, the frequency of searches for new antibiotics worldwide has increased because of the serious problem of antibiotic resistance among microbes. The recent discovery of novel primary and secondary metabolites from taxonomically unique populations of actinomycetes suggests that these organisms could add a new dimension to research into microbial natural products. However, only sparse information is available on actinomycetes in solitary wasp mud nest soil—one of the most productive ecosystems with regard to the occurrence of novel microbial flora. Our results strongly support the idea that species of solitary wasp mud actinomycetes, capable of growing under selective conditions of salinity, temperature, and pH, possess a significant capacity to produce compounds with unique antibacterial activity. The search for new actinomycete species seems likely to lead to the discovery of potentially beneficial secondary metabolites. The results obtained from this work are promising, and merit further studies on the purification, characterization and identification of the active secondary metabolites.

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