# Exploration of antimicrobial compounds from *Streptomyces* S9 against a phytopathogen, *Corynespora cassiicola* ( Berk & Curtis)

**S. Shalini Devi and K. V. Bhaskara Rao***

## ABSTRACT

The present study was carried out to explore potential antimicrobial compounds from Actinomycetes against common plant pathogens. Actinomycetes were isolated from different environmental samples and screened for antifungal activity against *Corynespora cassiicola* (Berk & Curtis), the causative agent of target spot disease in tomato plants. Among the isolates screened by the dual culture plate assay, an isolate S9 exhibited highest percent *in vitro* growth inhibition (85.5%) against *C. cassiicola*. This isolate was identified as *Streptomyces albolongus* based on morphology, biochemical characteristics and 16S rDNA sequencing. Two antimicrobial compounds were extracted and purified from culture filtrate of potent isolate S9 by Thin layer chromatography TLC, column chromatography and High Performance Liquid Chromatography (HPLC) techniques. Fourier transform infrared spectroscopy (FTIR), Mass Spectroscopy and Nuclear Magnetic Resonance spectroscopic investigations revealed the identity of compounds as propyl ester of Octadec-9-enoic acid and 17-hydroxy, 27-methoxy natamycin. The Minimum Inhibitory Concentration (MIC) of these compounds against several plant pathogens was recorded. Pure compounds exhibited broad spectrum antimicrobial activity. *In vivo* studies in tomato plants revealed the efficacy of purified compounds in controlling target spot disease caused by *C. cassiicola.*

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## INTRODUCTION

*Corynespora cassiicola* (Berk & Curtis) is the fungus, which belongs to the family of Corynesporascaceae and genus *Corynespora*. It is a globally distributed foliar plant pathogen with wide host range including more than 530 plant species from 380 genera. It affects many agriculturally important crops and economically prominent plants by causing leaf spot, target spot and leaf fall diseases (A-Li *et al.,* 2014; Looi *et al.,* 2017). Vegetable crops like tomato, cow pea, soybean, cucumber are drastically affected by this foliar pathogen (Dixon *et al.,* 2009; Lisboa *et al.,* 2016). Commercial plantations like rubber, cassava and cotton are severely infected with *C. cassiicola* leading to alarming economic losses (Pu Jinji *et al.,* 2007; Augusto and Fernando, 2016). It also causes stem blight disease in a medicinally important plant, *Phyllanthus amarus* (Mathiyazhagan *et al.,* 2011). The visual appearance of ornamental plants is affected by the leaf spot disease caused by *C. cassiicola* (Zaher *et al.,* 2005). Leaf spot disease of scarlet sage is also caused by *C. cassiicola* (Toshiko, 2008).

Anti-fungal agents like azoxystrobin, mancozeb and fumoxate provide excellent control of *C. cassiicola* (Ken *et al.,* 2002). However, recent investigations reveal that most of the registered fungicides failed in controlling target spot by *C. cassiicola* (Sheila *et al.,* 2013, Aveline *et al.,* 2013). The reason behind this failure can be the resistance developed by this pathogen to these fungicides as proved by research reports (Hagan *et al.,* 2013; Miyamoto *et al.,* 2010). In this scenario, only a few research reports describe eco-friendly methods to control this devastating pathogen and much of the research was confined to *in vitro* assays (Riddech *et al.,* 2017).

Remarkable research on different species of actinomycetes evidenced that secondary metabolites secreted by them constituted valuable bioactive compounds (Shalini *et al.,* 2015). Extending host range of *C. cassiicola,* its resistance to existing chemical measures and demerits of agrochemicals have motivated the present study to isolate antagonistic *Streptomyces*, extract and purify the active metabolites and evaluate their in vitro and in vivo inhibitory activity against *C. cassiicola*, a notorious phytopathogen.

## MATERIALS AND METHODS

### Isolation and screening for potent Actinomycetes

Soil and water samples for actinomycetes isolation were collected from different locations in Andhra Pradesh, India which include Tirumala hills (Lat 13º65'N, Long 79 º 34'E), Talakona (Lat 13 º 81'N, Long 79 º 21'E), Visakapatnam (Lat 17.72' N, Long 83º33'E) and Gundlakatta Manchi, Chittor district (Lat 13 º 19' N, Long 78 º 99'E). Isolation was done by serial dilution and spread plate technique (Amini *et al.,* 2016). Glucose-Yeast Extract Agar (GYA) and Actinomycetes Isolation Agar (AIA) were used for the isolation of actinomycetes. All the media were complemented with nalidixic acid (50µg/ml) to avoid bacterial contamination. After 7-14-days of incubation, morphologically distinct colonies (rough, chalky, wrinkled, powdery, earthy odour) (Khushboo, 2014) were selected and transferred to ISP7 agar plates. Plates containing pure cultures were stored at 4ºC until further investigation. Isolated actinomycetes were screened for antifungal activity by dual culture plate technique Riddech *et al.,* 2017). Each isolate was streaked on GYA plate as a line and incubated for 2-days at 28 ºC. A 9 mm disc of fungal pathogen was transferred to each antagonist plate and incubated for 5-7days at 28 °C. These plates were compared with control plate that contained fungal culture alone. Percentage of inhibition was calculated (Amini *et al.,* 2016).

### Collection and maintenance of phytopathogens

Phytopathogens *Pseudomonas syringae* (MTCC2730), *Colletotrichum capsici* (MTCC 10147), *Alternaria solani* (MTCC 10690), *Pythium aphanidermatum* (MTCC 10247) and *Corynespora cassiicola* (MTCC 2123) were obtained from MTCC, Chandigrah, India and maintained on Potato Dextrose Agar (PDA). *Xanthomonas malvacearum, Fusarium solani, Fusarium oxysporum, Cladosporium fulvum,* and *Rhizoctonia solani* were collected from the Citrus research station, YSR Horticultural University, Tirupathi. Andhra Pradesh. India.

### Taxonomic studies of isolate S9

Morphology of potent isolate S9 was studied by Scanning Electron Microscopy (S-3700N SEM). Cover-slip culture method was adopted to prepare the specimen for microscopic investigations. A thin layer of gold particles were coated on specimen before examining under SEM (Marianne *et al.,* 2012). Biochemical and physiological characteristics of isolate S9 were studied by following methods as described in previous reports (Reddy *et al.,* 2011). The DNA from S9 isolate was isolated and amplified by 16S rRNA primers. The amplified product was sequenced by a Sanger-based, automated sequencer (Eurofins genomics, Bangalore, India). The sequence was compared for similarity with the reference species (NCBI BLAST) and submitted to GenBank.

### Extraction, purification and structural elucidation of antimicrobial compounds

*Streptomyces* isolate S9 was inoculated in Cellulose Peptone Broth (pH 7) and incubated at 28ºC for 120-hrs in an incubator. The fermented broth was centrifuged at 8000 rpm for 10 min. Further the supernatant was added to an equal volume (1:1) of ethyl acetate and shook vigorously for two hours. Ethyl acetate layer was separated and concentrated using Rota evaporator. Antimicrobial activity of ethylacetate extract was tested by agar well diffusion assay (Prakash *et al.,* 2013).Thin layer chromatography was resorted for initial separation. Concentrated Ethyl acetate extract

was spotted on silica gel plate activated at 100ºC. Chloroform: ethanol: water (2:4:4) was used as mobile phase. The obtained chromatogram was placed in a sterile petri plate and overlaid with PDA containing spores of *C. cassiicola* and incubated for 5 days. The spot with active metabolites was scraped from the TLC plate, dissolved in 1ml of ethyl acetate, loaded in Sephadex LH-20 column and eluted with gradient solvent system consisting of hexane and ethyl acetate (0-100%). Elutions collected after column chromatography were concentrated and tested for antimicrobial activity. Active fractions (designated as AF1 andAF2) were further purified by preparative HPLC with C18 reverse phase column (Shimadzu LC-10 AVP) using 70% methanol as mobile phase. Structural elucidation of pure compounds was carried out by spectroscopical studies which included FTIR (Shimadzu FTIR 8400s), EI-MS, $^{1}$H NMR and $^{13}$C NMR (Bruker AMX 400MHz).

**Biological activity of active compounds**

Purified active compounds were diluted using DMSO to obtain concentrations ranging 1 µg/ml to100 µg/ml. Minimum inhibitory Concentration of antimicrobial compounds was determined by paper disc agar diffusion method (Cai *et al.,* 2008). Activity of the compounds was tested against various bacterial and fungal plant pathogens.

***In Vivo*** **disease control by purified compounds**

To evaluate the disease control potential of purified compounds, four treatments were tested on 6 week old tomato plants grown in plastic pots containing autoclaved peat mass and soil (1:1 ratio). Tomato seeds were sterilized with sodium hypochlorite for 15 min and washed thrice with sterile water. All plants were maintained at 23-26°C (day) and 20-22°C (night) with 15 hours of natural light and approximately 40% relative humidity. Six replications were maintained for all the treatments.Treatment-1: water as negative control, Treatment-2: chemical fungicide-Mancozeb (20 mg/ml), Treatment-3: compound 1 (20 mg/ml) and Treatment-4: compound 2 (20 mg/ml). Treatments were applied by spraying each solution. After drying the leaves, all the plants were inoculated with the spore suspension (3 x $10^4$ per ml) of *C. cassiicola.* Number of lesions developed was counted after 7-days and control efficacy was calculated.

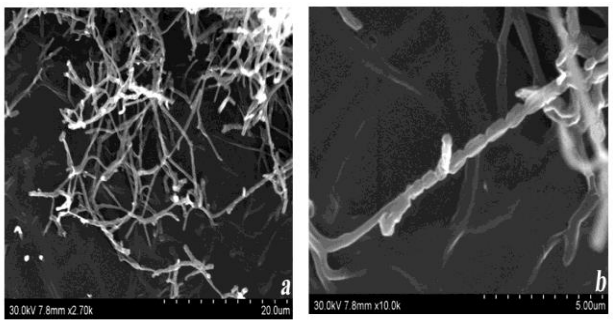
**Statistical analysis** The experimental data of *in vivo* studies was computed and subjected to ANOVA. Treatments mean were compared using the Tukey-Kramer Multiple Comparisons Test at $P<0.05$.

**RESULTS**

**Isolation and identification of the potential isolate**

A total of 24 actinomycetes were isolated and screened for antimicrobial activity against *C. cassiicola* by dual culture plate technique. Actinomycete isolate S9 from the rhizosphere soil presented strong inhibitory activity (85.56 %) than other isolates. Scanning Electron Microscopic observation of the isolate S9 showed a long mycelium and spore chains (8 - 10 spores in each chain) with open loops, primitive spirals and flexible hooks (Fig.1a and b). Microscopic and biochemical characteristics revealed that the isolate S9 belongs to genus *Streptomyces*. Phylogenetic analysis suggested that isolate S9 has a close genetic relationship with *S. albolongus* (LC128344) and the characteristics of isolate S9 were consistent with that of *S. albolongus*. So, based on the comprehensive phenotype and phylogenetic analysis, isolate S9 was identified as *S. albolongus.* The GenBank accession number of the 16S rDNA sequence of isolate S9 is KX247006.

**Fig1.** Scanning electron microscopic images of *Streptomyces* S9 (a) mycellium (b) spore chain

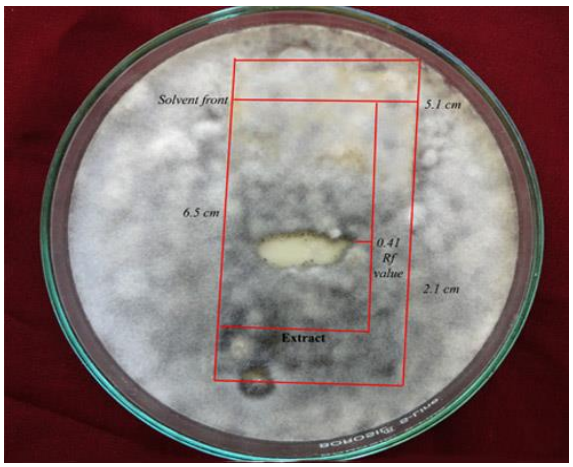


**Purification of bioactive compounds** **04**

About 1.8 g of ethyl acetate extract was obtained from 20 L of fermentation broth. *In vitro* inhibitory activity of ethyl acetate extract against *C. cassicola* (Fig. 2). Autobiography results showed the formation of inhibition zone around the spot with Rf value 0.41 (Fig. 3). During column chromatography a total of 20 fractions were collected among which two fractions exhibited antifungal activity when tested by well diffusion assay. These active fractions were further purified by preparative HPLC.

**Fig.2.** *In vitro* inhibitory activity of ethyl acetate extract from *Streptomyces* S9 against *C. cassicola*.

Fig. 3. Autobiography showing spot with antimicrobial activity against *C. cassiicola*




The FTIR spectrum of compound 1(obtained by purification of AF1) showed absorption peaks at 1242.2 , 1759.14 and 2995.5 which revealed the presence of functional groups esters (C-O), carboxylic acid (C=O) and alkane (C-H) respectively. EI MS analysis of Compound 1 indicated molecular weight of 324.3. $^{1}$H NMR (400MHz, Chloroform-d) of compound 1 showed protons at δ ppm 10.62 (s, 1 C) 13.99 (s, 1 C) 22.62 (s, 1 C) 23.97 (s, 1 C) 25.27 (s, 1 C) 28.71 (s, 1 C) 32.40 (s, 1 C) 34.35 (s, 1 C) 67.04 (s, 1 C) 130.53 (s, 1 C) 174.52 (s, 1 C) and $^{13}$C NMR (100MHz, Chloroform-d) exhibited peaks at δ ppm 10.62 (s, 1 C) 13.99 (s, 1 C) 22.62 (s, 1 C) 23.97 (s, 1 C) 25.27 (s, 1 C) 28.71 (s, 1 C) 32.40 (s, 1 C) 34.35 (s, 1 C) 67.04 (s, 1 C) 130.53 (s, 1 C) 174.52 (s, 1 C) (Fig. 4a and 4b). Based on the above data compound 1 was characterized as propyl ester of Octadec-9-enoic acid. ($C_{21}H_{40}O_2$). Its structure was indicated in Fig 5.

Fig. 4. $^{1}$H NMR (upper) and $^{13}$C NMR (lower) spectra of Compound 1 produced by *Streptomyces albolongus* S9

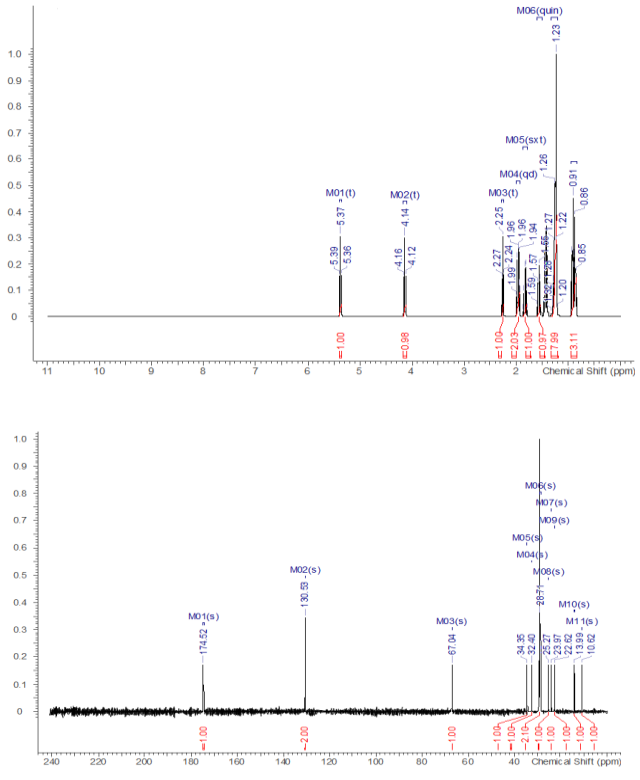
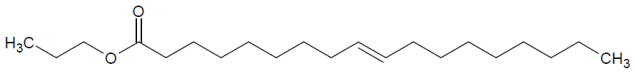
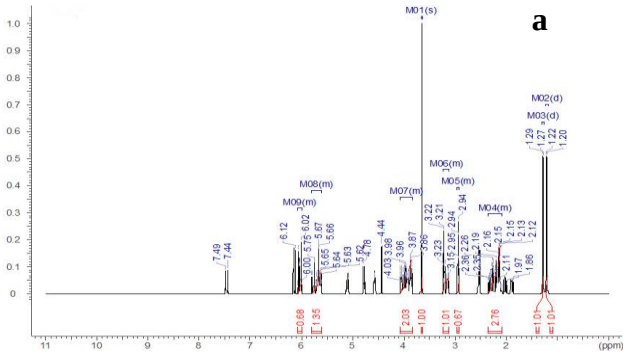



Fig. 5. Structure of propyl ester of Octadec-9-enoic acid (Compound 1)



The FT-IR spectrum of compound 2 (obtained by purification of AF2) showed the presence of peaks at 1718, 1565 and 1200-1120 which corresponds to ester, primary amine and -C-O groups respectively. Mass spectrum of compound 2 suggested that the molecular weight is 681.3. Structural elucidation of this compound was done by $^{1}H$ NMR and $^{13}C$ NMR (Fig. 6a and 6b). $^{1}H$ NMR (400 MHz, Chloroform-*d*) of Compound 2 depicted protons at δ ppm 1.21 (d, *J*=6.45 Hz, 1 H) 1.28 (d, *J*=6.71 Hz, 3 H) 1.88 (ddd, *J*=14.50, 5.37, 2.15 Hz, 1 H) 2.01 (ddd, *J*=14.57, 8.39, 7.39 Hz, 1 H) 2.08 - 2.36 (m, 8 H) 2.53 (dd, *J*=7.92, 4.70 Hz, 2 H) 2.92 - 2.96 (m, 2 H) 3.12 - 3.24 (m, 3 H) 3.66 (s, 3 H) 3.84 - 4.06 (m, 6 H) 4.44 (d, *J*=2.95 Hz, 1 H) 4.57 (ddd, *J*=6.18, 3.76, 1.88 Hz, 1 H) 5.07 - 5.14 (m, 1 H) 5.61 - 5.80 (m, 4 H) 5.99 - 6.08 (m, 2 H) 6.08 - 6.17 (m, 1 H) 7.46 (dd, *J*=15.85, 3.76 Hz, 1 H) and $^{13}C$ NMR (100 MHz, Chloroform-*d*) exhibited picks at δ ppm 17.39 (s, 1 C) 19.79 (s, 1 C) 35.19 (s, 1 C) 37.97 (s, 1 C) 41.97 (s, 1 C) 52.40 (s, 1 C) 54.52 (s, 1 C) 56.55 (s, 1 C) 66.20 (s, 1 C) 68.12 (s, 1 C) 69.89 (s, 1 C) 72.29 (br dd, *J*=54.20, 16.11 Hz, 1 C) 74.65 (s, 1 C) 101.43 (s, 1 C) 119.42 (s, 1 C) 129.31 (s, 1 C) 130.98 (s, 1 C) 132.06 (s, 1 C) 134.91 (s, 1 C) 136.02 (s, 1 C) 167.00 (s, 1 C) 172.04 (s, 1 C). Based on NMR data compound 2 is identified as 17-hydroxy, 27-methoxy natamycin ($C_{34}H_{51}NO_{13}$) (Fig. 7).

Fig. 6. (a) $^{1}H$ NMR and (b) $^{13}C$ NMR spectra of Compound 2 produced by *Streptomyces albolongus* S9



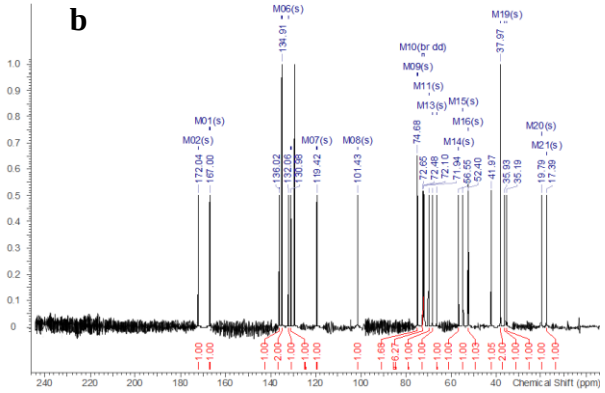
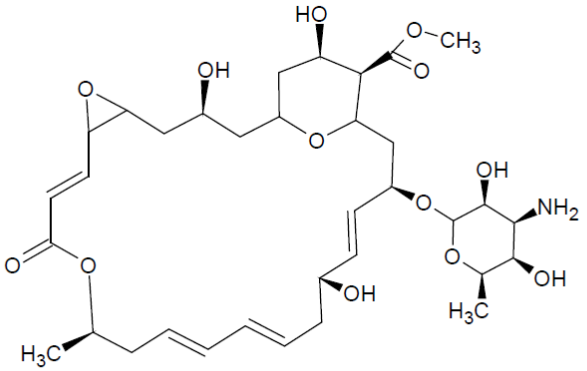




**Fig.7.**Structure of 17-hydroxy,27-methoxy natamycin (compound 2)




**Minimum Inhibitory Concentration**

Purified antimicrobial compounds from *Streptomyces* S9 isolate exhibited antibacterial and antifungal activities (Table 1). MIC of Compound 1 was higher than Compound 2. MIC of Compound 1aganist fungal pathogens ranged from 60-85 (µg/ml) where as Compound 2 ranged from 30-45 (µg/ml). MIC of Compound 1 against *P. syringae* and *X. campestris* is 10 µg/ml and 15 µg/ml respectively while MIC of Compound 2 against is 4 µg/ml and 6 µg/ml respectively.

***In vivo* inhibition of *C. cassiicola* infection**

The *in vivo* effect of isolate S9 metabolites on target spot diseases of tomato caused by *C. cassiicola* is shown in Table 2. Significant disease suppression (P<0.05) was observed in plants that received chemical fungicide (T2) or purified metabolites (T3 and T4). This is evident from disease index percentage which is significantly higher in control plants (T 1). Disease control efficacy of compound 1 and 2 was recorded as 78.4 % and 80.70%, respectivel.

**Table 1.** Minimum Inhibitory concentration (MIC) values of purified compounds against phytopathogens. The data are the average of six replications ± SE

| Pathogens | Compound 1 (µg/ml) | Compound 2 (µg/ml) |
|---|---|---|
| *Pseudomonas syringae* | 10±0.56 | 4±0.47 |
| *Xanthomonas malvacearum* | 15±0.57 | 6±0.76 |
| *Corynespora capsici* | 60±0.66 | 30±0.71 |
| *Alternaria solani* | 80±0.76 | 40±0.62 |
| *Pythium aphanidermatum* | 85±0.85 | 35±0.81 |
| *Colletotrichum cassiicola* | 60±0.60 | 40±0.76 |
| *Fusarium solani* | 70±0.66 | 35±0.72 |
| *Fusarium oxysporum* | 70±0.88 | 35±0.63 |
| *Cladosporium fulvum* | 80±0.66 | 45±0.71 |
| *Rhizoctonia solani* | 65±0.52 | 30±0.54 |

**Table 2.** *In vivo* Target spot disease control efficacy of purified compounds from *Streptomyces* S9 isolate.

| Treatments | Disease index (%) | Control efficacy (%) |
|---|---|---|
| T1- Water | 93.33±1.85 a | |
| T2- Mancozeb (20 mg/ ml) | 16.60±2.15 b | 82.70 |
| T3- Compound 1 | 20.11±1.8 b | 78.40 |
| T4- Compound 2 | 18.61±1.93 b | 80.70 |

The data are the average of six replications ±SD. Data in the same column followed by the same letter are not significantly different (P<0.05) according to analysis of variance.

## DISCUSSION

Biological control and related compounds produced by actinobacterial isolates are highlighted in many research reports (Khushboo *et al.,* 2014; Amini *et al.,* 2016). In the present research work, a potent biocontrol agent *S. albolongus* S9 has been isolated from rhizospheric soil.

Autobiography analysis revealed that spot with Rf value 0.41 possessed antifungal activities. Thus, this method helped pinpointing the exact bioactive fraction. These results are in agreement with the report of Prakash *et al.* (2013). Data from chromatographic and spectroscopic analysis resulted in detection of two antimicrobial compounds *viz.*, propyl ester of Octadec-9-enoic acid and 22-[(3-amino-3,6-dideoxy-β-Dmannopyranosyl) oxy]-1,3, 17, 26 tetrahydroxy-12- methyl-10-oxo-6,11,28-trioxatricyclo [22.3.1.05,7] 27methoxy, octacosa-8,14,16,18,20-pentaene-25carboxylic acid (17-hydroxy, 27-methoxy natamycin) from *S. albolongus* S9. Antifungal potential of fatty acids was elaborated in many review articles (Carolina *et al.*, 2011). Antifungal fatty acids are known to increase the membrane fluidity by disrupting phospholipid acyl chains. Presence of methyl or functional groups further enhances the antifungal activity of fatty acids as these groups occupy more

cross section in the membrane (Avis and Belanger, 2001). Hence, it is assumed that propyl or ethyl groups of octadec-9-enoic acid contributes for better antifungal activity in the present work. Crude fatty acid extracts from *Streptomyces* spp. were known to inhibit the biofilm forming *Streptococci* (Rajalakshmi *et al.*, 2014). Esters of Hexadecanoic acid and Octadecanoic acid extracted from Piper betle were known to inhibit *Streptococcus mutants* (Nalina and Rahim, 2006).

A novel polyene compound detected in this research is 17-hydroxy, 27-methoxy natamycin, an ester derivative of natamycin. Derivatives of natamycin were known to be more soluble than parent compound (Eric, 1999). This property may widen the antibiotic application. Natamycin was found to exhibit a broad spectrum antiphytopathogenic activity (Cai *et al.*, 2008). Very recently in 2015, activity of natamycin against various gram positive bacteria, gram negative bacteria, unicellular and filamentous fungi was recorded (Atta *et al.*, 2015). Species of *Streptomyces* so far explored for natamycin production included *S. lydicus, S. natalensis, S chattanovgensis* and *S. gilvosporeus* (Aparicio *et al.*, 2003*).* So far, no research report has documented the production of natamycin or its derivatives from *S. albolongus.*

Compounds extracted from various *Streptomyces* spp. are known for their *in vivo* efficacy in controlling plant pathogens. Phenylacetic acid and sodium phenyl acetate from *Streptomyces humidus* reduced the disease severity caused by the species of *Phytophthora* in pepper plants (Byung *et al.*, 2001). In the present study, spot disease in tomato plants caused by *C. cassiicola* was noticed to be successfully managed by application of purified compounds from *S. albolongus* S9 *viz.*, propyl ester of Octadecanoic acid and 17-hydroxy,27-methoxy natamycin. These results encourage further research on screening the soil contaminated with agricultural or industrial wastes for antimicrobial compound production that may result in cost-effective and eco-friendly micro biocide development.

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