

Antifungal potential of purified 3-(4-isopropylstyryl)-5-methylcyclohex-2-enone from marine actinobacteria *Streptomyces albus* A18

Mizaeir Mehtha Abdul Kader¹, Murugan Sambantham²
and Jothiprakasam Vinoth^{1,2,*}

¹Thiagarajar College. Department of Zoology & Microbiology. Madurai-625009. Tamil Nadu. India. *Email: vinodbio89@gmail.com.

²Annamalai University. Parangipettai-608 502. Tamil Nadu. India.

Abstract. Actinomycetes are known to produce potential secondary metabolites which comprise biological activity. The present work endeavor is to assess the fungicidal property of novel marine actinobacterial compound 3-(4-isopropylstyryl)-5-methylcyclohex-2-enone extracted and isolated from *Streptomyces albus* AC18. The crude compound was loaded on silica gel column and eluted with chloroform - methanol - water. The purity of isolated compound were analyzed by TLC using chloroform and methanol as the solvent system and verified by GC-MS. The purified compound structure was established from infrared, ultraviolet, ¹H-NMR, ¹³C-NMR and mass spectral data. The chemical shift assignments for the aliphatic compound from ¹H-NMR corresponds to molecular formula as C₁₈H₂₂O. The Bioassay-guided fraction leads to the isolation of compound, was identified as 3-(4-isopropylstyryl)-5-methylcyclohex-2-enone. Hence, this marine isolated *S. albus* AC18 actino-bacterial compound seem to be more efficient in its antifungal activity and acts as prominent reservoir for novel drug molecules en route for answering several fungal diseases.

Keywords: *Streptomyces albus*; Anti-fungi; TLC; GC-MS; NMR.

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ORCID

0000-0001-7042-8828
Mizaeir Mehtha Abdul
Kader

0000-0003-2769-4017
Murugan Sambantham

0000-0002-7706-1712
Jothiprakasam Vinoth

Introduction

Actinomycetes are most economically and biotechnologically valuable prokaryotes (Saravanakumar et al., 2010), possess capabilities in production of antibiotics and other compounds of biotechnological importance (Jain and Jain, 2005). Moreover they are

potential producers of antifungal compounds used as commercial antibiotics such as Natamycin “polyene” produced by *Streptomyces lydicus* AZ-55 (Atta et al., 2015). *Streptomyces* sp DPTB16 strain produced antifungal compound named 4’ phenyl-1-naphthyl-phenyl acetamide showing broad spectrum activity, finds application in managing human infectious fungal disease (Dhanasekaran et al., 2008).

Keeping in mind, the current investigation was attempted in isolation of potential antifungal compound 3-(4-isopropylstyryl)-5-methylcyclohex-2-enone from *Streptomyces albus* AC18 and to characterize its effect against fungal pathogens.

Materials and methods

Isolation of antifungal compound producing marine actinobacterial strain AC18

The sediments were collected from Pichavaram mangroves marine zone (Latitude 11° 25’ N and Longitude 79° 47’E) and transferred to the laboratory, stored at 4 °C. The collected sediments were land air dried aseptically. The serially diluted samples were plated on starch casein agar supplemented with 75 µg/mL of nalidixic acid and 80 µg/mL of cycloheximide (Himedia, Mumbai, India) prior to minimize bacterial and fungal growth and incubated at 28 °C for 8 days (Kim et al., 1994). After 8 days, morphologically different actinobacterial colonies were sub-cultured on ISP 2 medium slants and were maintained at 4 °C for further studies.

Screening of marine actinobacterial strain AC18 for antifungal activity

The isolated actinomycetes were screened for antifungal activity against *Mucor* sp fungal pathogen in cross streak plate method on Kuster’s agar, incubated at 28 °C ± 2 °C for 3 to 4 days (Kim et al., 1994). After observing a good ribbon like growth of the actinomycetes, the fungal pathogens were streaked at right angle near the original streak of actinomycetes and incubated at 28°C ± 2 °C for 48 h. The maximum clear zone colonies were considered as potential strain for actinobacterial compound production and characterization studies. As well control plates were also maintained to assess normal pathogenic fungal growth.

For second screening, the mature spores of *S. albus* AC18 strain were inoculated in 100 mL of fermentation medium containing Casein enzymic hydrolysate - 20g/L, Cystine - 0.5g/L, Sodium chloride - 5g/L, Sodium sulphate - 0.5g/L, Phenol red - 0.017g/L, Agar 3.5g/L, Final pH - 7.5 ± 0.1 and incubated on 30 °C for 8 days at 200 rpm. The fermented broth was centrifuged at 10,000 *xg* (4 °C) for 20 min and supernatant was filtered through 0.45 µm pore size membrane filter used for further antifungal activity (Ruan, 1977). To check antifungal spectrum, pathogenic fungus were cultured on Sabouraud dextrose broth at 27 °C for 24 h and consecutively swapped on Muller Hinten agar plates. Besides, the wells prepared at the plate’s center were filled with 100 µL of fermented medium and incubated at 27 °C for 3 days and the diameter of inhibition zone was observed.

Taxonomic identification of actinobacterial strain AC 18

The potential antifungal compound producing actinobacterial strain AC18 were identified based on morphological, biochemical and chemo taxonomical characteristics (analysis of cell wall sugar and cell wall amino acid analysis). Further utilization of carbon sources such as starch, dextrose, fructose, maltose, and mannitol and nitrogen sources namely D-alanine, L-arginine, and L-tyrosine were tested on Kuster’s agar.

Molecular identification and phylogenetic analysis

DNA was isolated from the pure culture of isolate as per protocol described by (Kieser et al., 2000). In brief actinomycetes spores were centrifuged at 13,000 *xg* for 5 min

and 1 μL of supernatant was used as template for amplification along with ITS 5 primer pair (White et al., 1990). The PCR conditions were as follows: initial denaturation at 95 °C for 15 min; followed by 45 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 1 min, extension at 72 °C for 50 s; and final extension at 72 °C for 10 min. PCR amplification was carried out using action specific forward primer, 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), used with reverse primers 1492R (5'-GGTTACCTTGTACGACTT-3'). The DNA sequences obtained from both strands were read on an ABI PRISM 377 DNA sequence. The homology of the sequences were analyzed using BLAST algorithm (<http://www.ncbi.nlm.nih.gov>) and were aligned with references taxa along with their GenBank accession numbers using Clustal W implemented in MEGA4 software (Tamura et al., 2007)

Purification and characterization of antifungal compound

Thin layer chromatography. TLC was performed to obtain partially purified compound. Crude sample were placed on the TLC plate as a single line and the chromatogram was made with the solvent system (Chloroform: Methanol). After separation, the active spot band was scrapped, mixed with methanol and centrifuged at 3,000 $\times g$ for 15 min. Followed by, supernatant was collected in a pre-weighed vial and kept under evaporation. Further, the partially purified compound obtained through TLC was tested for its antifungal activity against pathogenic fungi by well diffusion method.

FT-IR analysis. The partially purified sample was subjected to FT-IR spectroscopy to analyze functional groups such as sulphate, amino carboxyl and hydroxyl groups, (Perkin-Elmer-FT-IR-instrument). Briefly, the sample was mixed with 1,000 mg of dried KBR pellet of 3 mm diameter and 1 mm thickness. Absorbance spectra were recorded using Nicolet Avatus 360FTIR spectrometer equipped with KBR beam splitter and an air cooled DTGS detector. The absorption of light intensity of the peaks was calculated using the base line method; the frequencies for all sharp bands were accurate to 0.01 cm^{-1} .

GC-MS analysis. Consecutively to identify the compound, the sample was dissolved in methanol and subjected to GC-MS (Agilent technologies 6890N network GC system for gas chromatography). The analysis conditions were 20 min at 100 °C, 3 min at 235 °C for column temperature, 240 °C for injector temperature, helium was used as carrier gas and split ratio was 3:8. The sample (1 μL) was evaporated in a split less injector at 300 °C with the run time of 22 min. The component was identified through gas chromatography coupled with mass spectrometry. Further, the compound was identified through interpretation on mass spectrum using National Institute Standard and Technology (NIST) library search database which contained more than 62,000 drugs formulation. Hence mass spectrum of the unknown component was compared with the spectrum known components stored in the NIST08 and Wiley 08 library.

Nuclear magnetic resonance (^{13}C and ^1H NMR). NMR measurements were done at ^1H and ^{13}C spectra and recorded at 300 K on Bruker Advance DRX300, DPX400, DRX600 and DMX600 NMR spectrometers. Using standard Bruker software all 1D and 2D spectra was obtained. 3D structure of the pure compound was predicted based on NMR spectral data model using Chem3D Ultra software (Version 8). The samples were dissolved in CDCl_3 solvents and the residual solvent signals were used as internal standards (reference signal). The observed chemical shift (α) values are given in ppm and the coupling constant (J) in HZ.

Results

Isolation, identification of antifungal compound producing marine actinobacteria

A total of 22 actinobacterial strains were isolated from Pichavaram marine sediments and screened for its antifungal activity. Among them, the strain AC18 exhibited wide spectrum of antifungal activity against fungal pathogen. The vegetative mycelia of the strain AC18 grew abundantly on both synthetic and complex media, the aerial mycelia grew on International Streptomyces Project-7 medium (ISP-7), shown in. The SEM examination revealed the spore chains orientation owning warty surface. Neither both sclerotic granules and sporangia nor flagellated spores were observed. Chemo taxonomical analysis revealed that cell wall hydrolysate contains LL-diaminopimelic acid (LL-DAP) and the sugar pattern was not detected.

Table 1. Morphological and biochemical characteristics of actinomyces isolates.

Test	Growth characteristics
Morphological characteristics	
Spore morphology	Orientation
Colour of aerial mycelium	Grey
Colour of substrate mycelium	Coffee brown
Spore mass	Grey
Biochemical characteristics	
Indole production	Negative
Methyl red	Negative
Voges proskauer	Negative
Citrate utilization	Positive
H ₂ S production	Negative
Nitrate reduction	Negative
Catalase	Positive
Oxidase	Negative
Melanin production	Negative
Starch hydrolysis	Positive
Gelatin hydrolysis	Positive
Lipid hydrolysis	Positive
Casein hydrolysis	Positive
Haemolysis	Negative
Triple Sugar iron	Alkaline butt/Alkaline slant
Chemotaxonomic characters	
Whole cell sugar analysis	Negative
Cell wall amino acid analysis	LL-DAP
Carbon source utilization	
Starch	Highly positive
Dextrose	Highly positive
Fructose	Positive
Maltose	Highly positive
Mannitol	Highly positive
Nitrogen utilization	
D-alanine	Highly positive
L-arginine	Highly positive
L-phenylalanine	Highly positive
L-tyrosine	Highly positive

The morphological and biochemical investigations of isolate AC18 were presented in Table 1. The isolate AC 18 have utilized all carbon and nitrogen sources. The urease and catalase were shown positive, melanin production and citrate utilization were shown negative. The cultural characteristics of AC18 strain revealed that the growth depends on available nutrients present in the medium and the physical conditions. Upon the growth of the isolate AC18 on various media, Tyrosine agar (ISP-7) was observed to be a superlative medium for maximal growth (Table 2).

Table 2. Maximal growth on different medium.

Name of the medium	Aerial mycelium	Substrate mycelium	Pigmentation
Malt extract and yeast extract Agar (ISP-2)	Dull white	Dark yellow	Nil
Oat meal agar (ISP-3)	Grey	Red	Dark pink
Inorganic Salt Starch agar (ISP-4)	Grey	Yellowish green	Nil
Glycerol asparagine's agar	Grey	Light Grey	Nil
Tyrosine agar (ISP-7)	Grey	Brown	Nil
Kuster's agar	Grey	Dark yellow	Pale pink
Actinomycetes isolation agar	Grey	Brown	Nil
Starch casein agar	Grey	Brown	Nil

Based on the International Key's (Buchanan and Gibbons, 1974) and numerical taxonomy of *Streptomyces* species program (Hensyl, 1994) the AC18 strain seemed to be closely related to *Streptomyces albus*. Further, the molecular phylogeny of strain AC18 through 16S rDNA sequence and phylogenetic tree analysis confirmed that it has exhibited 99% homology with *Streptomyces albus*.

Production and purification of the antifungal compound from *S. albus* AC18

For the production of antifungal compound, fermentation was carried out in antibiotic production medium for 7 days at 27 °C followed by filtration through centrifugation at 5,000 $\times g$ for 15 min at 4 °C. After fermentation, the cell free supernatant was mixed with equal volume of ethyl acetate, and the solvent layer was separated using a separating funnel and concentrated in rotary evaporator at 80 °C. Among the five fractions obtained through thin layer chromatography (TLC), fraction III exhibited wide spectrum antifungal activity. For the analysis of respective fraction preparative thin layer chromatography (TLC) was performed which yielded a single spot with Rf value of 0.83.

Characterization of the antifungal compound

FT-IR spectrum depict that the strong absorption observed at 1,666 cm^{-1} was due to C=O stretching frequency and the peaks obtained at 2,870, 2,745 and 2,959 cm^{-1} were due to the presence of aliphatic C-H stretching and aromatic ring C-H stretching, respectively (Figure 1). Further, GC-MS analysis confirmed that the molecular weight of the purified compound was about 254 MW which were presented as 253 due to a proton loss (Figure 2).

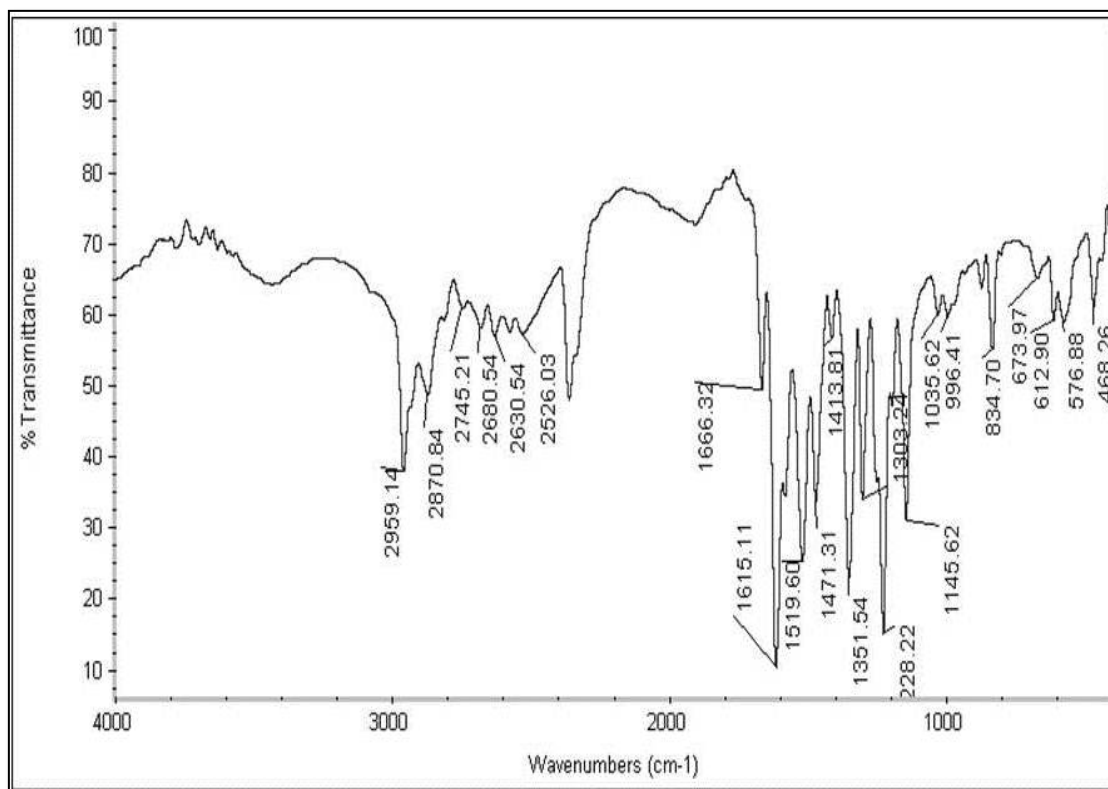


Figure 1. FT-IR spectrum of the antifungal compound from *Streptomyces albus* AC18.

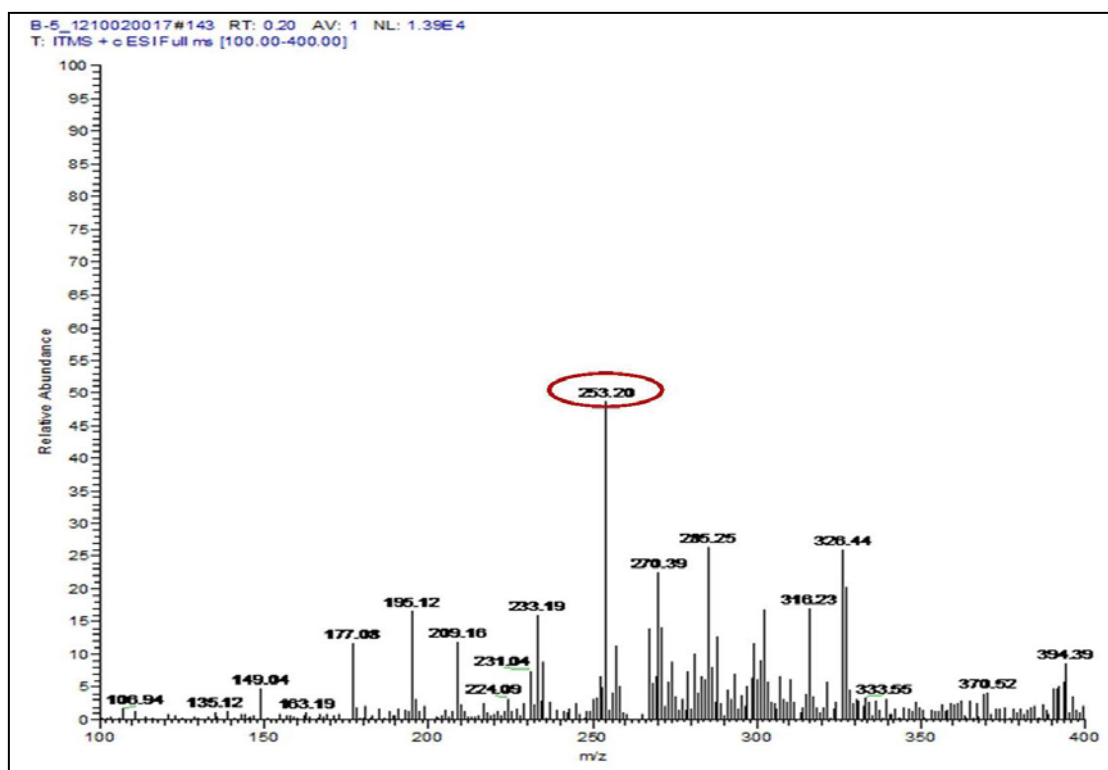


Figure 2. Mass spectrophotometry of the purified compound from *Streptomyces albus* AC18 with the molecular weight of 253.

Based on $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ studies, the purified antifungal compound from *Streptomyces albus* AC18 was identified as 3-(4-isopropylstyryl)-5-methylcyclohex-2-enone having molecular formula of $\text{C}_{18}\text{H}_{22}\text{O}$ and the molecular weight was about 253.20 MW. Structure of the purified antifungal compound 3-(4-isopropylstyryl)-5-methylcyclohex-2-enone produced by *Streptomyces albus* AC18 (Figure 3). 3D structure of the pure compound of 3-(4-isopropylstyryl)-5-methylcyclohex-2-enone (Figure 4).

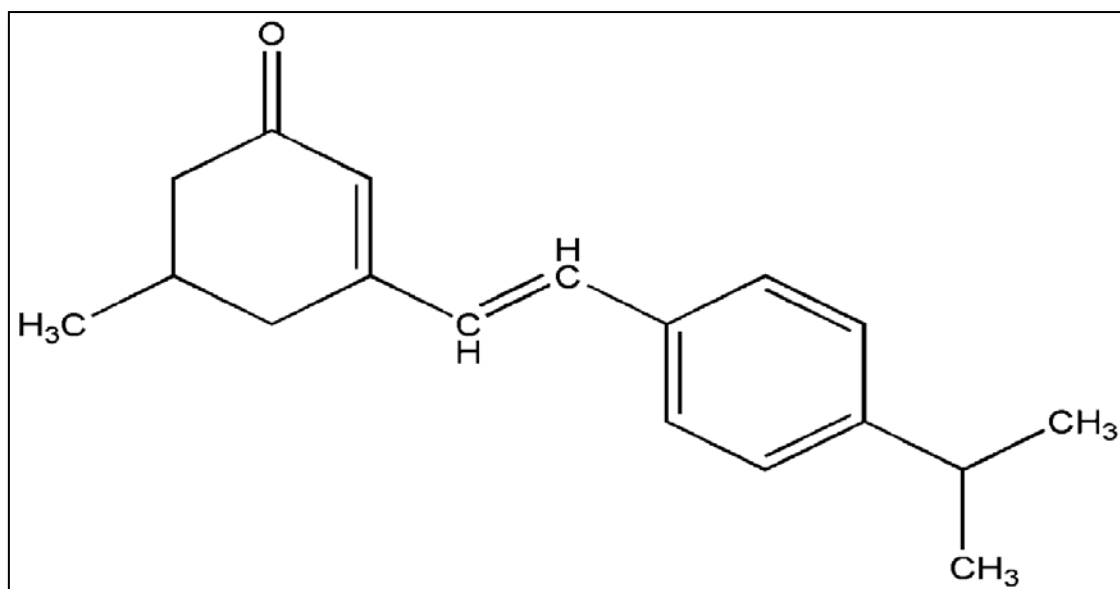


Figure 3. Structure of the purified antifungal compound 3-(4-isopropylstyryl)-5-methylcyclohex-2-enone.

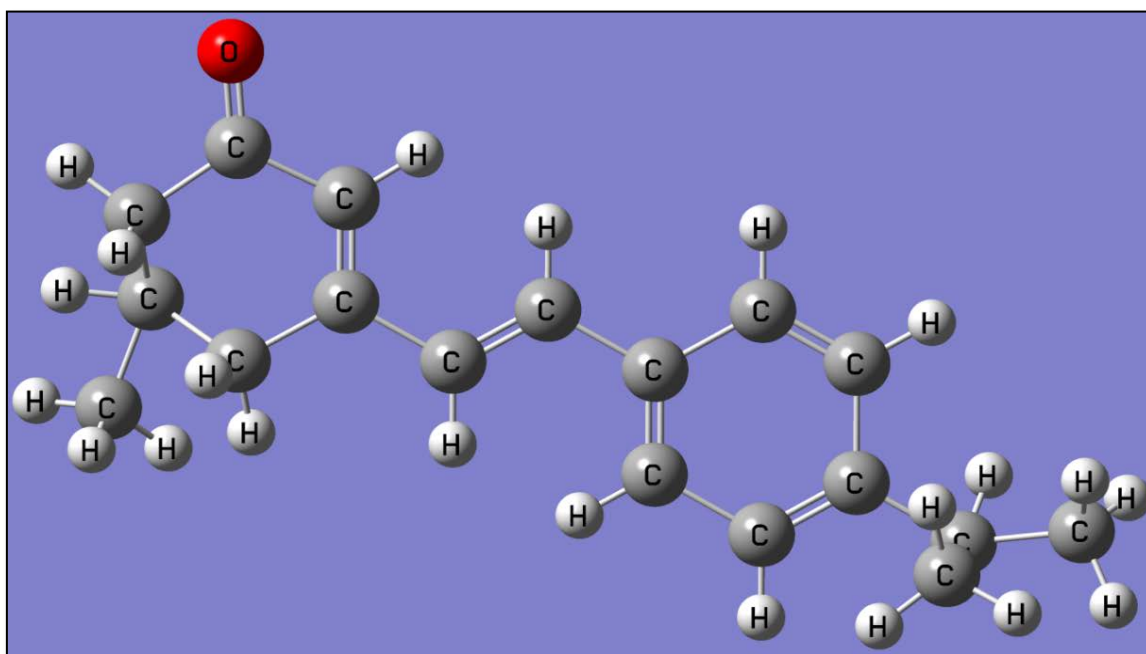


Figure 4. 3D structure of the pure compound of 3-(4-isopropylstyryl)-5 methylcyclohex-2-enone.

Discussion and conclusion

Fungal infections has been prominent in recent years and become a significant medical problem, due to increased immune-compromised patients with innate or acquired immune-deficiencies (Jarvis, 1995). Such increased frequency of multi-resistant pathogenic fungi have created demand in pharmaceutical industries in screening of new antibiotics with a broad spectrum of activity, which resist the inactivation processes exploited by microbial enzymes (Motta et al., 2004). Nevertheless several reports on control of secondary metabolite production by actinobacteria (Motta et al., 2004), pharmacological compounds production from marine actinomycetes with antibiotic and antitumor properties (Suthindhiran and Kannabiran 2004) are available. Hence search for active secondary metabolites produced by environmental isolates using poorly explored microorganisms, could provide a new source for novel bioactive compounds discovery.

In present study, a total of twenty-two actinobacterial strains were isolated from Pichavaram marine sediments and strain AC18 was found exhibiting wide spectrum antifungal activities. The maximum antifungal compound biosynthesis can be recorded at pH 7.0 (Atta, 1999); temperature 35 °C (Kunnari et al., 1997; Atta, 1999); glucose was found to be best carbon source (Hoshino et al., 2004) KNO₃ seem to be best nitrogen source (Hosokawa et al., 1996; Atta, 1999; Khalifa, 2008). The molecular taxonomy of actinobacterial strain based on 16S r DNA sequencing and the phylogenetic tree (diagram) revealed the identification of *Streptomyces albus* strain.

In this work, the antifungal compound extracted from culture supernatant was tested against fungal pathogens. The antifungal activity percentage was found higher when compared with the activity of Moroccan soil *Streptomyces* sp (Barakate et al., 2002). Moreover, strain AC18 antifungal activity was relatively different from other studies exhibiting 16% in soil of Turkey (Oskay et al., 2004); 53%-61% in Algerian soil (Sabaou et al., 1998) and 44.5% in soils of South-Eastern Serbia (Ilic et al., 2005, 2007). Whereas, similar inhibitory pattern was demonstrated by ethyl acetate extracts of marine *Streptomyces* sp. RM17 and RM42 (Remya and Vijayakumar, 2008) and marine *Streptomyces* isolates from the Andaman Coast of Bay of Bengal (Peela et al., 2005). The broad antifungal spectrum exhibited by strain AC18 highlighted its potential and proposed to be important candidate for antibiotics. Hence, the present study provides first hand information on antifungal agent production from *Streptomyces albus* against fungal pathogens. The identification of antifungal compound produced by strain AC18 was investigated through TLC, PTLC, FT-IR, GC-MS and NMR analysis. The results of TLC analysis confirmed the presence of single compound with R_f value of 0.55. Further, the purified compound was allowed to pass through silica gel column and eluted with chloroform and methanol (1:9 ratio); the R_f value of most active fraction against shrimp fouling fungus was about 0.83. Similarly, many workers purified the antifungal compounds through silica gel column and eluted with solvent comprised of various ratios of chloroform and methanol (Criswell et al., 2006; Sekiguchi et al., 2007).

The Numerical Magnetic Resonance (NMR) based studies revealed that the antifungal compound extracted and purified from *Streptomyces albus* AC18 was identified as 3-(4-isopropylstyryl)-5-methylcyclohex-2-enone (C₁₈H₂₂O) having the molecular mass of 253.20. The elemental analysis studies confirmed the ¹H-NMR and ¹³C-NMR spectrum of 3-(4-isopropylstyryl)-5-methylcyclohex-2-enone, the singlet appeared at 0.92ppm was due to methyl proton attached to C5 carbon. The potential antifungal compound named 4'-phenyl 1-naphthyl phenyl acetamide from *Streptomyces* sp. DPTB16 were identified and reported by (Dhanasekaran et al., 2008). However, Hwang et al. (2001) reported that, the phenyl acetic acid and sodium phenyl acetate are potential compounds obtained from *Streptomyces humidus*.

The present investigation reveals the importance of obtaining novel antifungal compound from *Streptomyces albus* AC18 against fungal pathogens. As a result the antifungal compound 3-(4-isopropylstyryl)-5-methylcyclohex-2-enone (C₁₈H₂₂O) produced by putative *Streptomyces albus* AC18 isolated from marine sediments might be used as novel antibiotics against fungal infections.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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