Chapter 5C

Purification and characterization of Antifungal compound from Streptomyces sp. A6
INTRODUCTION

*Streptomyces* have several key features that make them excellent candidates as biocontrol agents. Besides mycelia mode of growth which helps in efficient root colonization, they have been reported for production of variety of extracellular enzymes and range of antimicrobials (Crawford et al., 1993). Antimicrobials produced by *Streptomyces* have been implicated as one of the major factors attributing natural suppressiveness to soil against number of soil borne pathogens (Andrade, 1994). Many antibiotics from actinomycetes have been used directly or assumed to be responsible for the biocontrol potential of the producing strain. Examples of such metabolites include macrolide benzoquinones, aminoglycosides, polyenes, and nucleosides (Trejo-Estrada, 1998). In particular, about 60% of the antibiotics developed for agricultural use have been isolated from *Streptomyces* (Tanaka and Omura, 1993). Most of the antibiotics isolated from *Streptomyces* are antibacterial in nature while reports on antifungal metabolites are limited. Since, in agriculture, fungal plant diseases are more prevalent than bacterial there is a constant need for search of newer antifungal metabolites. The rate of discovery of new bioactive compounds from terrestrial actinomycetes has decreased (Fenical et al., 1999), actinomycetes from pristine habitats still provide opportunity for isolation of novel antibiotics. Marine microorganisms have been the topic for the investigation of number of natural products. As marine environmental conditions are extremely different from terrestrial ones, it is surmise that marine actinomycetes have different characteristics from those of terrestrial counterparts and, therefore, might produce different types of bioactive compounds (Selvin et al., 2013). The isolate *Streptomyces* sp. A6, isolated from intertidal zone of Arabian sea at Diu, exhibited antifungal activity against *Fusarium udum*. Thus the present investigation was carried out to purify and characterize antifungal compound produced by the isolate.

5C.2. MATERIALS AND METHODS

5C.2.1. Inoculum and culture conditions

The International Streptomyces Project medium II (ISP-II) containing (g/l): Malt extract, 10.0; yeast extract, 4.0; glucose, 4.0; pH 7.2 was used for growth and antifungal metabolite production. The medium was sterilized by autoclaving at 10 psi for 20 min. *Streptomyces* sp. A6 was grown at 30°C under shaking conditions (180 rpm) for 96 h.
5C.2.2. Antifungal potential of *Streptomyces* sp. A6 against fungal phytopathogens

The antifungal activity of *Streptomyces* sp. A6 against *Fusarium udum*, *Rhizoctonia solani* and *Aspergillus parasiticus* was determined by dual culture technique. *Streptomyces* sp. A6 culture plug of size 1 cm was placed in the centre of ISP-II agar medium, fungal plugs were then placed at an equal distance of 3 cm from the central plug and the plates were incubated at 28°C for 72 h. Zone of growth inhibition indicated the antifungal activity of isolate.

5C.2.3. Determination of non-protein nature of antifungal metabolite

The non-protein nature of antifungal metabolite in the culture supernatant was determined by protease K treatment, TCA precipitation, boiling for 30 min and autoclaving at 15 psi for 20 minutes. The residual antifungal activity was measured against *Fusarium udum* after each treatment. 0.5 mg/ml of protease K units (Sigma, USA) was used for treatment. For TCA precipitation 10 ml of 96 h grown culture supernatant was precipitated with equal volume of 5% TCA. The precipitate thus obtained was centrifuged at 10,000 rpm for 10 min and supernatant was assayed for its residual antifungal activity.

5C.2.4. Extraction of antifungal metabolite

Solvent used for extraction of antifungal metabolite were selected on the basis of Snyder polarity index (SPI). As the polarity of the solvent increases, the SPI also increases. The solvents used in the study were n-hexane (SPI=0), xylene (SPI=2.4), benzene (SPI=3.0), n-butanol (SPI=3.9), chloroform (SPI=4.1), ethyl acetate (SPI=4.3), acetone (SPI=5.4), acetonitrile (SPI=6.2), methanol (SPI=6.6) and chloroform+methanol (SPI=8.2). For extraction culture supernatant was mixed with equal volume of solvent and shaken vigorously for 30 min. The organic phase was collected, evaporated to complete dryness in vacuum evaporator and finally suspended in 1/10 of the original volume in the same solvent. The concentrate was then assayed for its antifungal activity by agar well diffusion method. A standard cork borer of 8 mm was used to cut a well in the center on surface of potato dextrose agar plate. Three agar blocks (8 mm diameter) containing confluent growth of the test fungus (*Fusarium udum*) was placed at equal distance from the central well, 100 µl of the solvent extracts were introduced into the central well and was allowed to diffuse for 30 min at 4°C and was then incubated in an upright position at 25°C for 72 hrs. Zones of inhibition
were measured and % inhibition calculated by comparing the zone of inhibition obtained with the extracts to that of respective solvent controls. The zone observed in case of the control is not exactly a zone of ‘inhibition’, however in the stipulated incubation time it is the maximum distance to which the fungus can grow in the absence of any inhibitory agent. This distance is arbitrarily considered as 0% inhibition. The distance of the well from the plug (3 cm) is considered as 100% inhibition i.e. the situation in which the fungus does not grow at all.

5C.2.5. Purification of anti-fungal compound by Thin Layer Chromatography

0.25 mm thick silica gel plates were prepared using 40% emulsion of Silica Gel GF254 (Qualigens). The plates were allowed to air dry and were subsequently baked at 60°C for 10-12 h (overnight). 10 pi of sample was applied to silica Gel GF254 plate and was eluted using different solvent systems based on Snyder polarity index: butanol (SPI=3.9), water saturated butanol (SPI=4.3), acetonitrile (SPI=6.2) and Butanol saturated water (SPI=8.6). The spots were visualised under UV transilluminator and concentration antifungal metabolite was estimated by densitometry using AlphaEaseFC software version 4.0.

5C.2.6. FTIR analysis of antifungal metabolite

The infrared spectra of active TLC fraction were recorded on Shimadzu FTIR-8400S model. The spectra were obtained using potassium bromide pellet technique. Potassium bromide was dried under vacuum at 100 °C for 48 hrs and 100 mg of KBr with 1 mg of sample was taken to prepare the pellet. The spectra were scanned in the range of 400-4000 cm⁻¹. The spectra were plotted as intensity versus wave number.

5C.2.7. GC/MS analysis of the antifungal metabolite

The active ethyl acetate extract was subjected to gas chromatography-mass spectrometry (GC-MS) analysis on Perkin Elmer Auto System XL Gas Chromatograph at SICART facility, V.V Nagar, Anand. The peaks of the gas chromatography were subjected to mass-spectral analysis. The spectra were analyzed from the available library data.
5C.3. RESULTS

5C.3.1. Antifungal activity of Streptomyces sp. A6

Streptomyces sp. A6 exhibited antifungal activity against three different fungal phytopathogens, *Fusariumudum, Rhizoctoniasolani* and *Aspergillusparasiticus* as revealed from dual culture technique by agar diffusion method (Figure 5C.1).

![Antifungal activity of Streptomyces sp. A6](image)

Figure 5C.1: Antifungal activity of *Streptomyces* sp. A6 against (A) *Fusariumudum*, (B) *Rhizoctoniasolani* and (C) *Aspergillusparasiticus*.

5C.3.2. Non-protein nature of antifungal metabolite from Streptomyces sp. A6

Antifungal metabolite in the culture supernatant of *Streptomyces* sp. A6 retained 91.2 ± 4.14, 89.57 ± 3.2, 88.55 ± 5.99 and 81.43 ± 3.23% activity after treatment with proteinase K, TCA precipitation, boiling and autoclaving, respectively revealing its non-proteinaceous nature.

5C.3.3. Extraction and purification of antifungal metabolite from Streptomyces sp. A6

Best extraction of compound was achieved with benzene (SPI=3.0) followed by n- butanol (SPI=3.9) > chloroform (SPI=4.1) > ethyl acetate (SPI=4.3) (Figure 5C.2). The compound was then purified by thin layer chromatography. The three spots obtained on TLC showed similar Rf values as that of spots obtained from Streptomyces fradiae (MTCC321) extract.
which was used as reference (Figure 5.C.3). All the three spots were scraped from the TLC plate and suspended in chloroform. Silica was removed by centrifugation followed by filtration through 0.2 micron nylon filter. Chloroform was evaporated to dryness and the antifungal metabolite was then dissolved in known volume of chloroform and subjected to antifungal assay by well diffusion method. The lower spot with Rf value of 0.46 possessed antifungal activity.

Figure 5C.2: Extraction of antifungal compound from culture supernatant of *Streptomyces* sp. A6 with different solvents. Values in parenthesis indicate amount of antifungal compound extracted(pg/ml) and % inhibition in growth of *Fusarium udum*
Figure 5C.3: Thin layer chromatography of antifungal extract from (a) *S. fradiae* and (b) *Streptomyces* sp. A6.

### 5C.3.4. FTIR and GC-MS analysis of antifungal metabolite

The TLC purified antifungal metabolite was subjected to FTIR and GC-MS analysis. Figure 5C.4 shows the FTIR spectra of the antifungal metabolite produced by *Streptomyces* sp. A6 which was similar to that of antifungal metabolite from *S. fradiae*. The absorption peaks obtained from IR spectra suggested presence of alkyl groups, C-H (2920, 2850, 1470 and 1260 /cm), aldehyde/ketone group, C=O (1720 /cm), nitro group, N-O (1390-80 /cm) and alcohol group, C-O (1020-60 /cm). GC analysis of crude and purified antifungal compound from *Streptomyces* sp. A6 revealed a common peak at 45.89 min (Figures 5C.5(a) and 5(b)) which was further subjected to MS analysis (Figure 5C.6). The molecular weight of compound was found to be approximately 440.
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Figure 5C.4: FTIR spectra of antifungal compound from (a) S. fradiae and (b) Streptomyces sp. A6.
5C.4. DISCUSSION

*Streptomyces* are known to produce number of antifungal agents active against widerange of fungal pathogens. These include mycolytic enzymes, siderophores and antifungal compounds (Crawford et al., 1993). The antifungal metabolites form *Streptomyces* are of great interest and have been used widely in the medical field for control of pathogenic infection. However, there use in agriculture, for control of fungal plant diseases is limited. Extremely high counts of streptomycyces from some naturally suppressive soils and ability to secrete antibiotics in sterile soils suggests their biocontrol potential against soil borne pathogens in field conditions. Biocontrolagents which produce antifungal compounds besides mycolytic enzymes can provide better protection against fungal phytopathogens as antifungal metabolites are generally more stable in varying temperatures and pH conditions and can persist for longer durations in fields. The antifungal compound extracted from *Streptomyces* sp. A6 was found to be non proteinic in nature and stable even after boiling for 30 minutes. Snyder polarity index (SPI) was used to select proper solvents for extraction and separation of antifungal compound from the culture supernatant of *Streptomyces* sp. A6. The Snyder Polarity Index is a measure of the ability of solvent to interact with various polar/non-polar test solutes; it also indicates the polarity of a solvent. On SPI scale water has highest SPI of 9.0 while n-hexane has a SPI of 0.0. Depending on the polarity of the compounds they are extracted to varying extents in solvents of different SPI. Extraction of antifungal compound from *Streptomyces* sp. A6 decreased significantly in solvents with SPI above 4.3 or below 3.0 on the SPI scale. Similarity in the Rf value of the antifungal compound extracted from *Streptomyces* sp. A6 to antifungal compound from *Streptomyces fradiae* MTCC321 suggested that the compound may be fradicin, an antifungal compound produced by *S. fradiae*. Since 16S rDNA sequence of *Streptomyces* sp. A6 exhibited 96% homology to *S. fradiae* and fradicin was not available commercially, a standard strain of *Streptomyces fradiae* MTCC 321 was processed in the same manner as *Streptomyces* sp. A6 for antifungal compound extraction and purification. Moreover the molecular weight of the antifungal compound as determined by GC-MS analysis was also found to be similar to that reported for fradicin. Fradicin was first reported by Swart in 1951 an antifungal compound produced by *Streptomyces fradiae*. Fradicin has been reported to have low solubility in water. The same was also evident from our studies as fradicin was better
extracted in non-polar solvents. Furthermore, fradicin has proved to be effective against number of fungal phytopathogens especially, *Pythium* sp., *Rhizoctonia* sp. and *Fusarium* sp, but has no activity against *Trichoderma* and *Rhizobium* sp. Ineffectiveness against *Trichoderma* and *Rhizobium* sp. is especially advantageous as many members of *Trichoderma* sp. are used as biocontrol agents against number of phytopathogens and members of *Rhizobium* sp are symbiotically associated with many leguminous plants. Soil application of fradicin had shown it highly stable in different soil types and soil pH (Gregory et al., 1952). However there were no studies on thermal stability of fradicin. Present study revealed that the antifungal compound from *Streptomyces* sp. A6 was highly stable at elevated temperature and retained 80% of its activity even after autoclaving. Broad spectrum antifungal activity, high stability in different soil types and stability at high temperatures suggests potential of fradicin as essential ingredient of antifungal preparations to control soil borne fungal phytopathogens.