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Extraction and Detection of Antimicrobial Compound from Streptomyces Filamentosus

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Abstract: **Streptomyces members are well known source of bio control agents due to their ability to produce various secondary metabolites like antibiotics and enzymes. In the present study, a soil actinomycetes member *Streptomyces filamentosus*was studied for its ability to produce antimicrobial compounds under controlled conditions. Optimum conditions such as type of medium, pH, temperature, incubation time, salt concentration, carbon and nitrogen source etc., for the production of antimicrobial compounds was determined. It was observed that it can produce two major antibiotic substances one resembles streptomycin ($C_{21}H_{39}N_7O_{12}$) and another was near dioxolomycin ($C_9H_{13}NO_4$). In the present study the conditions for the maximum production and the process of production, extraction was also studied. The structural details were determined using NMR, Mass spectroscopic and HPLC analysis.**

Keywords: **Secondary metabolites, compounds, NMR, Mass Spectroscopy and HPLC.**

1. INTRODUCTION

Actinomycetes produce more than half of the world's antimicrobials and are consequently becoming valuabletools in the field of biological control. Actinomycetes have been evaluated as a source of biocontrol agents or antibiotic compounds based on their distribution in various habitats[8] [3] [6] [21]. Saprophytic Gram-poitive bacteria in the genus *Streptomyces* have been shown to have charactristics, which make them useful as biocontrol agents against soil fungal plant pathogens.These characteristics include productionof different kinds of secondary metabolites and biologically active substances of high commercialvalues such as enzymes and antibiotics. Streptomyces are of the major contributors to the biological buffering of soil and have roles in decompositon of organic matter conductive to crop production.Besides, they have been studied as potential producers of antibiotics and exert antagonistic activity against wide range of bacteria and fungi [15] [10].

It has long been known that some of the actinomycetes strains of one species could generate different antibiotics, where as some other strains belonging to different species generate the same antibiotics [12] [13]. The production of antibiotics by actinomycetes therefore may not be species specific, but rather strain specific. Antibiotics originated from actinomycetes comprise a wide range of chemical structures, including aminoglycosides, anthracyclins, glycopeptides, betalactums, macrolides, nucleosides, peptides, polyenes, polychetides, actinomycines and tetracyclines.

2. MATERIALS AND METHODS

Extraction and detection of compound:

The fresh cultures of *S. filamentosus* were inoculated separately into each Starch Casein Broth flasks and incubated for 7 days at 28±2^{0}C shaking at 150 rpm maintaining at pH 7. After incubation the liquid was separated by filtration with Whatman No.1 filter paper. The filtrates from the two isolates were processed for solvent extraction for compound

recovery by using Ethylacetate. It was added to the filtrate in the ratio of 1:1 (v/v) and shaken vigorously for 1 h for complete extraction and extract was repeated twice.

The filter paper discs (8 mm diameter) were impregnated with dried ethyl acetate extract and placed on to the plates seeded with test organisms and activity was detected by Disc Diffusion assay [2]. The plates were then incubated at 28 ± 2^0C for 24-48 hrs and zone of inhibition was recorded. To test if the compound is intracellular or extracellular, the culture was centrifuged at 10,000 rpm for 20 min before filtration. The supernatant and biomass were extracted with ethyl acetate and tested for their antifungal activity using Well Diffusion method [2]. Ethyl acetate was used as control. The ethyl acetate phase that contains the compound was separated from the aqueous phase and evaporated to dryness in a water bath at 80^0C and stored at 4 ^{0}C for further analysis.

Detection of the antibiotic compound:

Determination of the compound by Thin Layer Chromatography:

The sample was subjected to Thin Layer Chromatography and analyzed on silica gel (40%) using methanol: ethyl acetate in 1:1 ratio as the solvent system. Compound was spot inoculated on to the TLC plate and was allowed to run in solvent system. The plate was then subjected to Iodine vapors and observed for spots on the TLC plate [15]. Rf value was calculated by the formula:

Rf = Distance travelled by the solute ÷Distance travelled by the solvent front.

Determination of the compound by Mass Spectroscopic assay:

The extracted antimicrobial compound in powdered form was analyzed for determination of its molecular weight by Mass Spectroscopy. Analysis was performed on Xevo G2-S which is designed for identifying and conform the modest range of compounds in a complex [19] [4][15]. This assay was performed with the assistance of Department of Chemical Sciences, IICT, Hyderabad.

Nuclear Magnetic Resonance (NMR) Spectroscopic Analysis to conform the structure:

The antimicrobial compound was analyzed for $C^{13}$and proton NMR to determine the number of carbon and hydrogen molecules and the covalent bonding between carbon and hydrogen present in the molecule to determine the structure of the component. The NMR spectrum was obtained using Homo Nuclear COSY H^1-H^1 Spectroscopy. The spectrum obtained was compared to get the structural details using Chem Draw software.

HPLC Assay:

The antibiotic compound from *S. filamentous* swas estimated by HPLC using a Nucleiosil C_{18} column in a Dionex Ultimate 3000 model semi analytical HPLC system. The compound was determined with the column eluted at a flow rate of 20 ul/ min with total run time of 30 min, using a gradient from 10-100% Acetonitrile in water. The absorption of solvent extracts *S. filamentosus* was detected by UV- Visible detector at 272nm. Concentration of the sample was determined by analyzing the peaks obtained [5] [20] [7] [14] [18].

3. RESULT

Determination of Production medium:

S. filamentosus were tested for the antibiotic activity in different media like SCB, GASp, OB, GYMB, NB, and MYEB. *S. filamentosus* has highest activity in SCB.NaCl concentration for maximum antibiotic compound production was observed by supplementing 1-9% NaCl to the SCB. Growth and production was observed from 1% to 9% with 1 % interval. It was observed that 5% NaCl concentration was found to be optimum for antibiotic activity of *S. filamentosus*.

Growth and metabolic activity was observed from pH 5-9 with intervals of 0.5. The best activity was observed at pH 6.5.*S. filamentosus* was inoculated into SCB and incubated at different temperatures in the range of 20^0C-55^0C with an interval of 5^0C. After seven days of incubation the culture filtrates of *S. Filamentosus* was tested for antifungal activity. The activity was found good at the range of 25-30^0C. Culture flask incubated at 28 ± 2^0C was the control at which the maximum activity was observed. So 28 ± 2^0C was optimum temperature for the growth and production of the antibiotic substance.

Effect of different carbon sources on antibiotic production by *S. filamentosus* was recorded by using Glucose, Starch, Maltose and Glycerol as carbon sources amending them individually into Starch Mineral Salts Agar (SMS) which was a basal medium. Among these carbon sources starch was found to be a good carbon source for *S. filamentosus.* The results were compared with the control i.e. activity of the two Actinomycetes in SMS Agar without any added carbon source. Effect of nitrogen source on antibiotic production of *S. filamentosus* was carried out by inoculating organisms in SMS medium by replacing nitrogen source (1%) with $NaNO_3$, KNO_3, $CaNO_3$, Peptone, NH_4OH, and Yeast extract. Each set of basal medium with different nitrogen sources was observed for the antibiotic production by Well Diffusion against the test bacteria. The best nitrogen source for both *S. filamentosus* was $NaNO_3$. Culture filtrate of SMS without carbon and nitrogen source (control) possesses very less activity, indicating less production of antibiotic compound.

Carbon and nitrogen sources were taken in the ratio of 0:5:0.5, 0.5:1.0, 1.0:1.0 and 1.0:0.5 % w/v into the basal medium to determine the suitable ratio of carbon and nitrogen source for maximum production of the antibiotic compound. Basal medium without carbon and nitrogen sources was taken as control for both the isolates. From the observations of Well Diffusion, C: N in the 1.0:0.5 ratios was found to be the best for the production.

Determination of incubation period for maximum production of antibiotic substance:

S. filamentosus inoculated and incubated in SCB from 0 to 15 days at 28 ± 2^0C. The culture filtrates were tested for antibacterial activity. Growth was observed from fifth day onwards and gradually increased by 7^{th} day after which the activity was found. The growth and activity was optimum at 7.0 days.

Extraction and detection of compound:

The fresh cultures of *S. filamentosus* was inoculated separately into each Starch Casein Broth flasks maintaining all the optimum conditions for high yield. Inoculated SCB flask was incubated for 7-10 days at 28^0C and good aeration was provided by shaking at 150 rpm. After incubation the liquid was separated by filtration with Whatman No.1 filter paper. Recovery of the Compound from the filtrates was by using ethylacetate. The filtrate was mixed with ethyl acetate in the ratio of 1:1 (v/v) and shaken vigorously for 1 h for complete extraction. The filtrate was extracted twice with ethyl acetate.

The activity was detected by Disc Diffusion assay [2]. Filter paper discs (8mm diameter) were impregnated with ethyl acetate extract, dried and placed on to the plates seeded with test organisms. The plates were then incubated at 28^0C for 24 h and zone of inhibition was recorded. The ethyl acetate phase that contains the compound was separated from the aqueous phase and evaporated to dryness in a water bath at 80^0C and stored at 4^0C for further analysis.

Detection of the antibiotic compound:

Determination of the compound by Thin Layer Chromatography:

The sample from *S. filamentosus* was subjected to Thin Layer Chromatography analysis on silica gel (40%) using methanol: ethyl acetate in 1:1 ratio as the solvent system [11]. Sample was spot inoculated and was allowed to develop for 2 h in chromatographic chamber. When the solvent front had reached the desired height, the strip was dried to drive off water and solvent and to fix the constituents in the strip. After standing for 2 minutes it was developed with Iodine vapors. Two spots were observed on the TLC plate. Rf value, the ratio of movement of the band to the total movement of the solvent was calculated for the two spots [17]. Rf value for spot one was 0.48 cm and for spot two 0.66 cm.

Molecular weight determination for the compounds by Mass Spectroscopic assay:

The extracted antimicrobial compound was analyzed for its molecular weight by Mass Spectroscopy [18] with the assistance of Department of Chemical Sciences, IICT, Hyderabad. Analysis was performed by using Xevo G2-S which is designed for identifying the modest range of compounds in a complex [9].

Mass spectral details for compound I of the ethyl acetate extract from S. filamentosus:

MASS SPECTRA:

Chemical Formula: $C_{21}H_{39}N_7O_{12}$

Exact Mass: 581.3

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Molecular Weight: 581.6 m/z: 581.27 (100.0%), 582.27 (23.6%), 583.27 (5.6%), 582.26 (2.6%) Elemental Analysis: C- 43.37; H- 6.76; N- 16.86; O- 33.01.

IUPAC Name of the above compound I:

1,-((1R,2R,3S,5R,6S)-4-((4R,5S)-3-((5R,6S)-4,5-dihydroxy-6-(hydroxymethyl)-3-(methylamino)tetrahydro-2H-pyran-2-yloxy)-4-formyl-4-hydroxy-5-methyltetrahydrofuran-2-yloxy)-2,5,6-trihydroxycyclohexane-1,3-diyl)diguanidine

Mass spectral details for compound II of the ethyl acetate extract from S. filamentosus:

MASS SPECTRA:

Chemical Formula: $C_9H_{13}NO_4$

Exact Mass: 199.1

Molecular Weight: 199.2

m/z: 199.08(100.0%), 200.09(10.0%), 201.09(1.3%).

Elemental Analysis: C- 54.26; H-6.58; N- 7.03; O-32. 13.

NMR spectroscopic analysis to conform the structure:

The antimicrobial compounds were analyzed for C^{13} and proton NMR studies to determine the number of carbon and hydrogen molecules and the covalent bonding between carbon and hydrogen present in the molecule to determine the structure of the compound. The NMR spectra were obtained using HomoNuclear COSY H^1-H^1Spectroscopy. The spectra obtained were compared to get the structural details using Chem Draw software.

The Chemical formula for each compound was recorded from the Mass Spectroscopy and NMR analysis. The compound I (Fig 1) from *S. filamentosus* was closely related to Streptomycin ($C_{21}H_{39}N_7O_{12}$) and compound II (Fig 2) resembles Dioxolomycin ($C_9H_{13}NO_4$).

HPLC Assay:

Representative solvent extracts of *S. filamentosus* were taken and chromatographed by semi analytical HPLC system. For the solvent extracts of *S. filamentosus* the column was eluted at a flow rate of 20ul/min with the total run time of 30 min, using a gradient from 10-100% acetonitrile in water. Quantity of the samples was determined by analyzing the peaks obtained in the chromatogram. The quantity of the Streptomycin related substance produced by *S. filamentosus* was 1.6076 µg /ml (Fig 3).

List of figures:

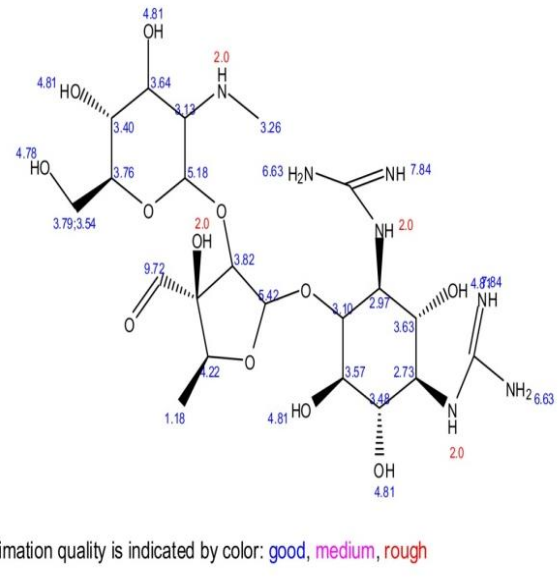

Fig.1: Chemical nature of compound I

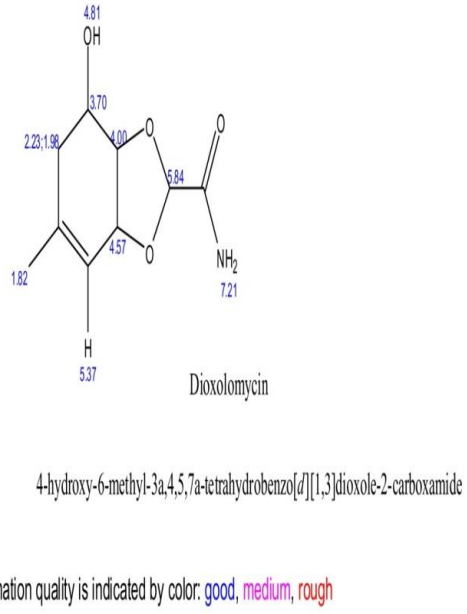

Fig.2: Chemical nature of compound II

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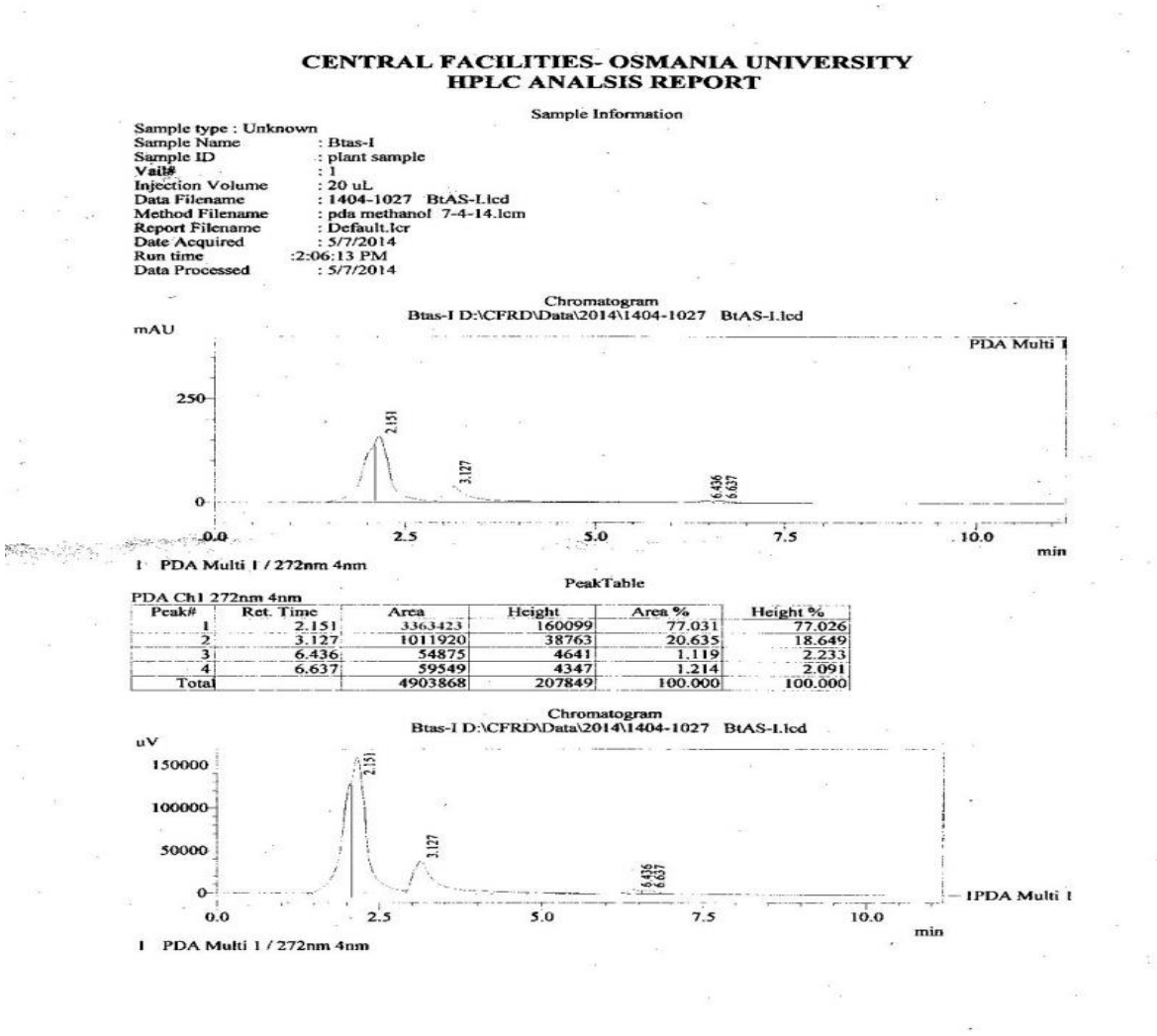

Fig.3: Chromatogram of the solvent extract of *S. filamentosus*

4. CONCLUSION

Actinomycetes have been evaluated as a source of biocontrol agents or antibiotic compounds based on their distribution in various habitats. Saprophytic Gram-poitive bacteria in the genus *Streptomyces* have been shown to have charactristics, which make them useful as biocontrol agents against soil fungal plant pathogens.These characteristics include productionof different kinds of secondary metabolites and biologically active substances of high commercialvalues such as enzymes and antibiotics.

The physiological conditions play an important role in the growth and production of the antibiotic compound by the organisms.Incubation temperature, pH, NaCl concentration, Carbon, Nitrogen sources, C/N ratio influence the metabiolic reactions, growth and production of the organisms. So, in the present research, the influence of physilogical conditions on the growth of S. filamentosus was determined. A temperature of 28^0C, pH 6.5, NaCl 5% concentration, Starch as carbon source, $NaNO_3$ as nitrogen source and C/N ratio at 1:1 were recorded optimum for the growth and production of the Actinomycetes.

The compounds from the extracts were further analyzed by Mass and NMR spectroscopy for their molecular characterization. The results revealed that the compounds from *S. filamentosus* were detected as analogues of streptomycin and dioxolomycin. The two compounds were structurally similar to the extracted compounds from *S. filamentosus*. This was reported to produce combination of antibiotics like glycopeptide antibiotics similar to streptomycin, which are active against Gram positive and Gram negative bacteria and another antibiotic named as

dioxolomycin which has cytostatic activity. The approximate Mass was 580.1 and 199.1 respectively for streptomycin and dioxolomycin analogues. The proton NMR revealed that it has a formula of $C_{21}H_{39}N_7O_{12}$, and $C_9H_{13}NO_4$.

Antibiotics originated from actinomycetes comprise a wide range of chemical structures, including aminoglycosides, anthracyclins, glycopeptides, betalactums, macrolides, nucleosides, peptides, polyenes, polychetides, actinomycines and tetracyclines [1], [16].

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