

Screening of *Streptomyces sp.* MTCC4 for the production of antifungal compound against *Sclerotium rolfsii*

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Abstract

Sclerotium rolfsii is a pathogenic fungus, infects about 500 plant species of about 100 families. *Streptomyces species* MTCC4 show remarkable activity against this fungus. The culture broth of *Streptomyces species* MTCC4 has inhibitory effect on the fungus in petriplate around the paper disc. Crude culture shows peaks at 2.8, 3.5, 3.8, 4.0, 5.4, 5.9 and 8.43 min in HPLC analysis at the 220 nm wavelength at the flow rate of 0.5 ml/min, in C18 column. After extraction and purification of each fraction, the fraction with retention time 4.1 min at the Flow rate of 0.5 ml/min and detection wavelength 310 nm, show maximum antifungal activity. 50% Methanol is used as mobile phase for HPLC analysis. Carbohydrate and protein test confirm the presence of carbohydrates and proteins. This compound is identified as a glycoprotein of 11.9 KD have antifungal activity against *S. rolfsii*.

Keywords: *Sclerotium rolfsii*, *Streptomyces sp.* MTCC4, Glycoprotein, Retention time.

Introduction

There are several harmful pathogenic fungi which have vast range of plant host. These pathogen remain in control by their natural enemies. The antagonistic compounds secreted by these organisms carried out significant reduction in the number of these fungi (El-Tarabily *et al.* 2000). Several studies are carried out to separate and identify antagonistic compound from producer organisms (Brigitte *et al.* 2008; Masami *et al.* 2008; Masayuki *et al.* 2008; Md. Zakir *et al.* 2008; Naoya *et al.* 2008; Sloan *et al.* 2008 and Stephen 2008).

The nature of these compound varied. It may be volatile compound, Antibiotic, peptide or an enzyme (Mahmoud 2000). *Sclerotium rolfsii* is a devastating plant pathogen infecting over 500 plants species in 100 families (Punja, 1985, Punja 1988, Sarma *et al.*, 2002

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and Azhar *et al.*, 2003). It attacks diverse phases of the development of hosts, from the seed to the agricultural products. It is able to survive and thrive within a wide range of environmental conditions. Growth is possible within a broad pH range 5 to 8.

The mycelium growth occurs between 20 and 35 °C (Azhar *et al.*, 2003). This fungus is able to produce sclerotia, which can survive for several years. The aim of present study was to screen out new strains of *Streptomyces* against *Sclerotium rolfsii*.

Materials and methods

All the chemicals required were obtained from Merck Co. and Himedia Co. The source of microbial strains was microbial type of culture collection (MTCC, IMTECH, Chandigarh, India). The culture was maintained in slant culture media of following composition: 10 gm/lit. Dextrose, 3 gm/lit. malt extract, 3 gm/lit. Yeast extracts, 5 gm/lit. Peptone and 20 gm /lit. Agar. The pH was maintained in between 6.4 - 6.8. Organism was cultured in nutrient broth media inoculated with 24 hr old seed culture maintained at 28°C and 80 rpm in rotatory orbital shaker. Inoculated nutrient broth media was incubated at 28°C for 144 hrs. pH of culture medium was 7.5 and centrifuged at 5000 rpm.

The culture filtrate was extracted three times by the same volume of ethyl acetate. After the removal of cell mass, supernatant was analysed by spectrophotometer at different wavelength. This was carried out to determine the optimum detection wavelength for HPLC analysis.

For the inhibition test 4% potato dextrose agar was used to grow the *S. rolfsii* in petriplate along with paper disc containing culture broth filtrate of *Streptomyces sp.* MTCC4. The same test was also performed on *Aspergillus niger* MTCC 872. Culture filtrate extracted by ethyl acetate was analyzed by HPLC. 10 µl of sample was injected to C18 column (250mm X 4.6mm X 5mm). The flow rate was 0.50 ml/min. Sample was analyzed at 220 nm wavelength. In order to purify the active fraction (methanol: chloroform 20: 80 extract); chloroform was added gradually (drop-wise) until formation of the first precipitate. The precipitate was separated by centrifugation. Each fraction was tested in inhibition test. Ammonium sulphate was also used for the extraction. The

ammonium sulfate was grind in a glass mortar to be a fine powder that easily to dissolve. This powder was added gradually to the antagonistic solution. The solution was shaken using a vortex. The formed precipitate was separated by centrifugation. The amount of ammonium sulfate was noted for each precipitate and calculated as saturation percent. Each precipitate was dissolved in water and re-precipitated again by ammonium sulfate as a purification step. (Shawn 1991) Each precipitate was dissolved in methanol and centrifuged to remove any residue of ammonium sulfate. This step was repeated several times until the removing all amounts of ammonium sulfate. Each precipitate was used for inhibition test and analyzed by HPLC. Carbohydrates were determined by phenol sulfuric method as described by Dubois et al. (1956).

3 ml of alcoholic 2 N KOH solution was added to a known weight of the pure compound in a test tube. The tubes was covered and left overnight for Saponification. The saponified sample is then neutralized by adding four ml of 2N sulfuric acid. The tube was shaken well. The free fatty acids were extracted by diethyl ether several times. The combined ether extracts were washed with water several times, and then evaporated to dryness at 40°C. The remaining fatty acids were then weight as described by Hamilton (1992).

Results

The inhibition test by using 10 ml of the culture filtrate showed a zone of inhibition around the paper disc. This indicates the presence of a considerable amount of the antagonistic compounds in this volume of culture filtrate (Fig 1).



Figure 1: Inhibitory zone around the paper disc in *S.rolfsii* culture plate.

The culture filtrate extracted by chloroform didn't show any inhibition effect. This reflects the polar fashion of the antagonistic compounds. Therefore, the culture filtrate was evaporated to dryness under vacuum and the remaining residue was extracted by different solvents with increasing polarity. The residue remaining after evaporation of methanol extract only showed inhibition effect against *S. rolfsii*. The spectrophotometer scanning for the methanol extract showed wavelength at 220 nm. That have the maximum absorbance. This wavelength was used as detection wavelength in HPLC analysis of this extract.

The optimum HPLC separation was obtained by using C18 column. Mobile phase: methanol. Flow rate: 0.50ml/ min. and detection wavelength 220 nm. The HPLC for the methanol extract showed presence of 7 compounds. Their retention times are 2.8, 3.5, 3.8, 4.0, 5.4, 5.9 and 8.43 which are the major compound (Fig 2). Purification of the active fraction of the previous test (methanol: chloroform 20: 80 extract) was carried out by adding chloroform gradually (drop-wise) until forming of a precipitate. The precipitate was separated by centrifugation.

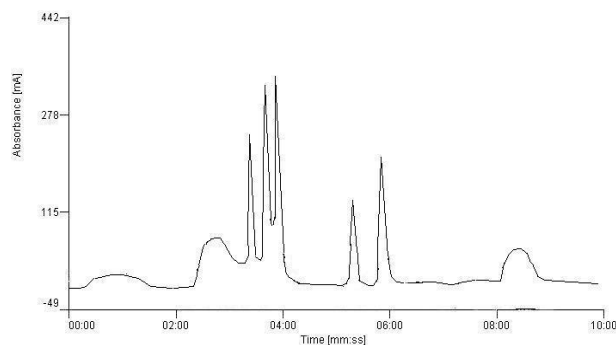


Figure 2: HPLC chromatogram of culture filtrate of *Streptomyces* sp. MTCC4:conditions are: C-18 column, 0.5 ml/min flow rate, detection wavelength 220 nm.

Three layers were formed. The upper was sticky, the middle was a powder and the lower was the chloroform solution. The upper layer only showed inhibition effect against *S. rolfsii*. HPLC analysis showed that the major two compounds were stile that having Rt 3.7 and 4.02min (Fig 3)

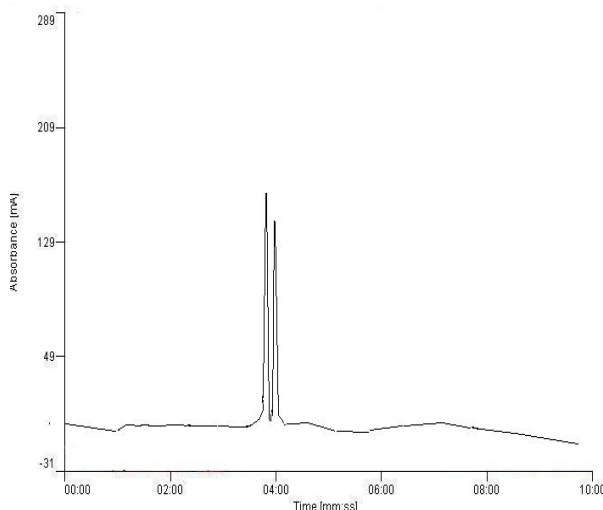


Figure 3: HPLC chromatogram of culture filtrate of *Streptomyces* sp. MTCC4:conditions are: C-18 column, 0.5 ml/min flow rate, detection wavelength 310 nm .

All of the fractions were analyzed by HPLC and used in inhibition test against *S. rolfsii* which were extracted by ammonium sulfate . The first precipitate only showed the maximum inhibition . As the compound was highly soluble in water, extracted from mixture by water. HPLC analysis of water extract showed retention time 4.1 min (Fig 4). The compound which show maximum activity against fungi had the retention time

of 4.1 min. This active glycolipoprotein (Rt. 4.1) was a sticky compound.

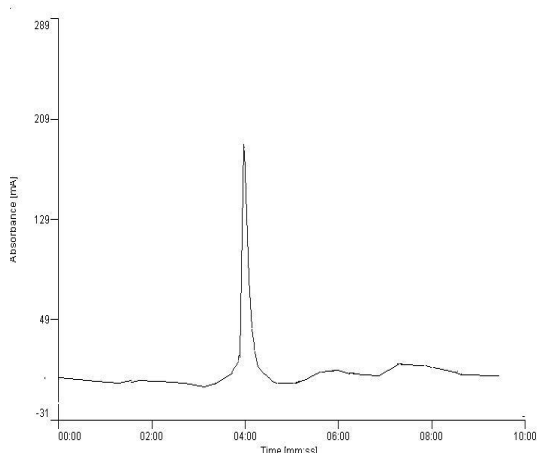


Figure 4: HPLC chromatogram of culture filtrate of *Streptomyces sp.* MTCC4 (water extract): conditions are: C-18 column, 0.5 ml/min flow rate, detection wavelength 310 nm.

It has a light brown color and has a maximum absorbance at 230 nm when dissolved in methanol. The glycolipoprotein nature of microbial products were found by other researchers such as Leila *et al.*, (1981) on *Pseudomonas aeruginosa* which is a human pathogen. The extracellular slime glycolipoprotein produced by this strain induces a tumor necrosis factor production by human monocytes. The analysis of this glycolipoprotein showed that it consists of Hexose 12%, Hexosamine 20%, Uronic acid 4%, Protein 18% and Lipid 30%. While the work obtained by Anastassiou *et al.*, (1987) about the slime material from *Pseudomonas aeruginosa* strain isolated from a patient with bacteremia, was found to contain 16% uronic acids, 48.5% carbohydrates, 11% protein and 2% lipids. The sugars contents were galactose, glucose, and ribose, with trace amounts of rhamnose and mannose. Lathigra *et al.*, (1996) reported that the pathogenic bacterium *Mycobacterium tuberculosis* produces a 19-kDa glycolipoproteins. George *et al.*, (2003) found that the slime glycolipoproteins of *Pseudomonas aeruginosa* consists of: neutral sugars 22.33%, Hexosamines 11.4%, Uronic acid 6.5%, Protein 15.8%. The sugars contents (g/100g total sugars) were glucose 36%, mannose 22%, galactose 25%, rhaminose 8.4%, fucose 5% and xylose 4%. This compound was mainly responsible for the antifungal activity. This compound was previously identified as glycoprotein in case *Bacillus pshychrosaccharolyticus*. The molecular weight of compound was 11.9 KD (Abdel azeiz *et al.*, 2009). As the HPLC analysis and inhibitory test give same results in both cases, the statement can be made that both compounds are identical in their nature. The comparison of these two organisms may give better organism to find more economic way for commercial production of antagonistic compound.

Conclusions

Current study gave results that *Streptomyces sp.* MTCC4 is producer of antagonistic compound. HPLC chromatogram retention time (4.1 min) and peak area showed that the concentration of compound was adequate. Inhibition test also showed positive results. There are several other microorganism which have this property. So a comparative study among these organism can give a better producer organism. The change in concentration of nutrient also affect the

concentration of compound in the culture broth. Different carbon and nitrogen sources can also enhance its production. This will lead to more economic commercialization of antagonistic compound.

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