

Extraction and Molecular Characterization of Antimicrobial Metabolites from *Streptomyces rochei* against Bacterial Leaf Blight of Cotton Caused by *Pantoea* sp.

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ABSTRACT

India ranks among the largest producer of cotton worldwide with high economic value. During the time of cultivation cotton plant normally suffers from the bacterial blight diseases, leading to colour change of affected leaves into light brown exhibiting a blighted appearance. While retrieving the causative agent on nutrient agar plate, yellow pigmented bacteria were consistently recovered which was thought to be caused by *Xanthomonas oryzae* pv. *oryzae*, the cotton bacterial blight pathogen. However, physiological and molecular analysis of isolated causative agent was of the bacterium *Pantoea* sp. eighteen strains of active antimicrobial metabolites producing *Streptomyces* sp. were isolated from the soil samples of agricultural field of Mehsana district, North Gujarat, India. All the isolates were assessed for antagonistic activity against *Pantoea* sp. A causative agent of bacterial blight disease of cotton plant. Among the strains tested, isolate Gray-1 showed strong antimicrobial activity. This isolate was identified as a *Streptomyces rochei* through genetic analysis. The antimicrobial compounds obtained from ethyl acetate extract of *Streptomyces rochei* were investigated using FTIR and Gas Chromatography-Mass Spectrometry (GC-MS). GC-MS analysis showed the presence 20 bioactive compounds. The most active compounds are 3-Hydroxy-2-Methylbenzaldehyde, 2-Methyl-3-Beta-Furyl propenal, 5-Endo-5-Exo-(Epoxy-methano)-6-Methylidene-7-oxabicycloheptane, 4-Hydroxy-Methylbenzaldehyde, 3-Hydroxy 4-Methylbenzaldehyde and 3,4 (Methylenedioxy) Toluene, 2-Hydroxy 5-Methylisophthalaldehyde.

Key words: Antimicrobial Metabolites, Bacterial Leaf Blight, GC-MS, *Pantoea* sp., *Streptomyces rochei*.

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INTRODUCTION

In India, cotton production possesses a serious threat being susceptible to suffer from variety of pathogenic diseases by different agents. Bacterial blight diseases caused by the pathogens of genus *Xanthomonas* have devastated different plants species, leading to large amount of losses in productivity as well as quality

of the plant harvests.^[1,2] The highly valuable crop is pressurized by diverse fungal and bacterial attacks too.^[3] Bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* is the most important and oldest known bacterial disease^[4] and the most serious bacterial diseases in many of the rice growing regions of the world.^[5] The bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* and also known to be caused by *Pantoea*.^[6,7] For the discovery of novel drug compounds which can actively act against wide range of targets, natural products can be used as a most consistent source for biologically active molecules with varied chemical/structural diversity.^[8] Microbes serve as the key producers of the secondary metabolites, continuing to be a useful source of novel secondary metabolites with a range of biological activities.^[9]

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Most useful and renowned antibiotics from microbial source were isolated from actinobacteria phylum of bacteria and more than 75% of commercially useful antibiotics are produced by different *Streptomyces* sp.^[10] Thus, screening, isolation and characterization of different strains of actinobacteria capable of producing potential antibiotics from different sources pose a major thrust area of research since many years.^[11]

In the present study, isolation of antimicrobial metabolite producing *Streptomyces rochei* with potent activity against *Pantoea* sp. has been explained in details with the relevant results. Additionally, production, purification, structure elucidation and bioactivity of the isolated antimicrobial metabolite from the source have also been discussed in depth with correlation.

MATERIALS AND METHODS

Isolation and fermentation of *Streptomyces rochei*

All antimicrobial metabolites producing actinobacteria's strains were isolated from the soil samples of an agricultural field of Mehsana District, North Gujarat, India. Pure cultures were maintained on SCA slants. Morphological, biochemical, cultural and physiological tests were performed according to the methods described in Systematic Bacteriology, Bergey's Manual. Species level identification of selected microbial strains was carried out at Biokart Pvt Ltd., India. The isolated culture of actinobacteria was inoculated in 250 mL of medium (10 g starch, 4g yeast extract, 2g peptone, 5ml potassium bromide (20 g/L), 5mL iron and sulphatetrahydrate (4.76 g/L), DW 1L, pH 6.5). All the flasks were incubated at 30°C and 150 rpm on a rotary shaker for 48 h, which were used as seed cultures. 100 µL of these seed cultures each was used to inoculate 250 mL of medium. The flasks were then incubated at 30°C on a rotary shaker at 150 rpm for 12 days. Antimicrobial activity was performed by using well diffusion assay against *Pantoea* sp.

Collection of infected leaves of cotton and isolation of causative bacteria

The infectious cotton leaves were collected from local farming area of Mehsana district, North Gujarat and used for the isolation. Sterile polythene bags were used to store infected cotton leaves at 4°C. Then the infected leaves were treated with 0.1 N HgCl₂ and crushed in sterile distilled water using mortar and pestle. 1 mL of 10⁻⁶ diluted suspension was spread on N-agar plates and further incubated at 30°C for 72 h. The primary cultures were isolated by using the spread plate technique on N-agar plates (HiMedia).^[12]

Extraction of antimicrobial metabolite

The cell biomass of fermented culture was separated using Whatman's filter paper with Buchner funnel. Ethyl acetate was added to the filtrate and kept for vigorous shaking for 1 h to extract crude product. The solvent phase which retained the crude metabolite was separated from the aqueous phase and concentrated at 40°C using an evaporator. The residue obtained was mixed in ethyl acetate for antimicrobial assays. Antifungal activities of the crude extracts were determined by using agar well diffusion assay.^[13] 1 ml of test organisms each was added in 20 ml of Muller Hinton molten agar tubes and poured onto plates. A diameter of 6 mm was prepared by using sterilized Cork borer to bore wells into the agar and 50 µL of the extract loaded into the wells. The extract was allowed to diffuse into the agar by keeping at stationary condition before the plates were incubated under aerobic conditions at 30°C for 24 h. The wells loaded with Streptomycin (50 µg/mL) served as a positive control whereas wells loaded with ethyl acetate served as negative control. All the plates were incubated at 30°C and zone of inhibition was measured after 72 h of incubation. The extracted crude powder were dried and used for further analysis.

FT-IR and GC-MS analysis

FTIR and GC-MS analysis is used for the analysis of crude ethyl acetate extract of *Streptomyces rochei*. FT-IR spectra were recorded on Bruker, Tensor 27 Infrared spectrometer. GC-MS analysis was performed by using GC Shimadzu QP2010 system and gas chromatography interfaced to a Mass Spectrometer (GC-MS) equipped with Elite-1 fused silica capillary column. As the carrier gas, Helium gas (99.99%) was used at a constant flow rate of 1.51 cm³/min with injection volume of 2µl (split ratio: 20). The NIST 2005 library, which is a software feature of the GC-MS data acquisition system, is utilized to confirm the identification of the components of the unknown sample.

RESULTS

Morphological and physiological Characterization of *Streptomyces*

The isolated strain of *Streptomyces* possesses the colonies with Circular, Convex and greyish colour characteristics. The various complex sources like casein and starch was utilized by bacterial culture for the growth. The isolated strain was also found to produce certain extracellular enzymes like protease, amylase and cellulose. The isolates were identified as a *Streptomyces rochei* and the Accession No. is MN114054. A total of 1152 nucleotide bases of

the strain were used to generate the phylogenetic tree as depicted in Figure. (Figure 1).

Isolation of *Pantoea* sp. and screening of *Streptomyces* for antimicrobial activity

The cotton plant leaves infected with bacterial blight disease are shown in Figure 2. The colonies of gram negative bacteria isolated from the infected leaves are shown in Figure 3. The isolated bacterium from infected leaf was identified as *Pantoea* sp.

Out of the total actinobacterial isolates, during the screening process, four isolates were found to have good antimicrobial activity. One of the isolates of *Streptomyces* sp. shows significant activity against infectious gram-negative bacteria with the zone of inhibition of 24 mm (Figure 4).

Molecular characterization of purified compound

Figure 5 presents the FTIR spectrum of ethyl acetate extract of *Streptomyces rochei*. Table 1 represents the data on the peak values and the probable functional groups present in the extracts. Based on the peak values of the FTIR spectrum, the region of IR radiation helps to identify the functional groups of the active

components present in extract. The results of FTIR analysis confirmed the presence of (C-H) Aromatic group; (OH) Functional group and N=N. Major peaks were observed at 3011.22 cm⁻¹, 3648.54 to 3675.15 cm⁻¹, 1141.53 cm⁻¹ and 1744.36 cm⁻¹. They could be assigned to the (C-H) Aromatic group, (OH) Functional group and N=N respectively.

The results pertaining to GC-MS analysis led to the identification of 20 compounds from the GC fractions of the ethyl acetate extract of *Streptomyces rochei*. Mass Spectrometry attached with GC was used for the identification of the compounds. The results obtained were shown in Table 2 and Figure 6. The gas

Table 1: FTIR analysis.		
Number of compound	Peak Value	Bond
1	3011.22 cm ⁻¹	(C-H) Aromatic group
2	3648.54 to 3675.15 cm ⁻¹	(OH) Functional group
3 (1)	1141.53 cm ⁻¹	N=N
3(2)	1744.36 cm ⁻¹	N=N

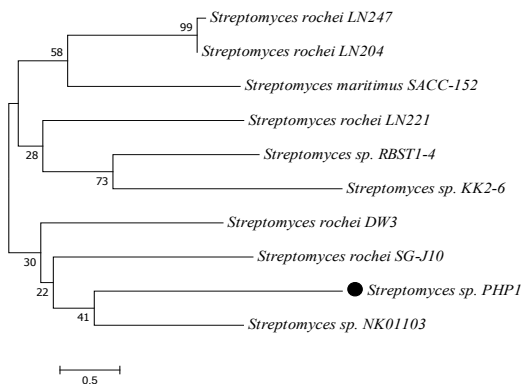


Figure 1: Phylogenetic tree of Grey-1.



Figure 3: Isolation of *Pantoea* sp.



Figure 2: Bacterial blight on cotton leaves.



Figure 4: Antimicrobial activity of *Streptomyces rochei*.

chromatogram obtained shows relative concentrations of various compounds getting fractionated at their specific retention time. The most of bioactive compounds found in the Ethyl acetate extract of *Streptomyces rochei* are 3-Hydroxy-2-Methylbenzaldehyde, 2-Methyl-3-Beta-Furyl Propenal, 6-Hydroxychroman-4-one, 2-Hydroxy-5-Methylisophthalaldehyde.

DISCUSSION

Total 18 strains of streptomyces were isolated from the soils of North Gujarat, on the other hand extracts of cotton leaves infected with bacterial blight disease were

spread on the nutrient agar added with soluble starch. Secondary metabolites extracted from *Streptomyces rochei* poured in to the wells for the analysis of action against infected bacteria identified as a *Pantoea* sp.

Pantoea agglomerans has been established as pathogen of cotton.^[14] It was isolated from a field in south Carolina and identified through 16s ribosomal DNA sequencing and phylogenetic tree construction^[15] reported boll rot of cotton caused by *Pantoea* sp. To be sure of pathogenicity, strains of *Pantoea* sp. were used to inoculate cotton^[16] and analyzed the deleterious effects of *Pantoea* sp. on plant. *Pantoea* sp. has been identified as a pathogen of cotton, maize, rice etc. Mechanisms involved in pathogenesis of plants are hrp system, phytohormones, quorum sensing

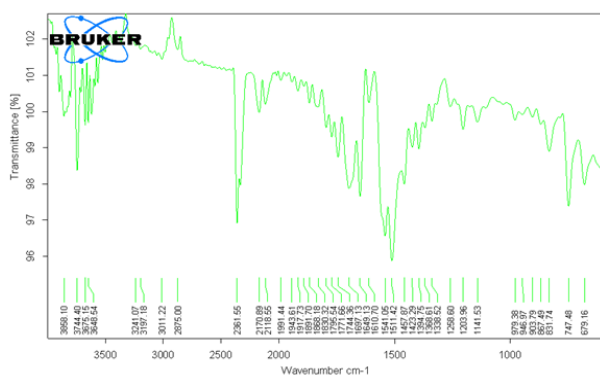


Figure 5: FTIR spectra of Ethyl acetate extract of *Streptomyces rochei*.

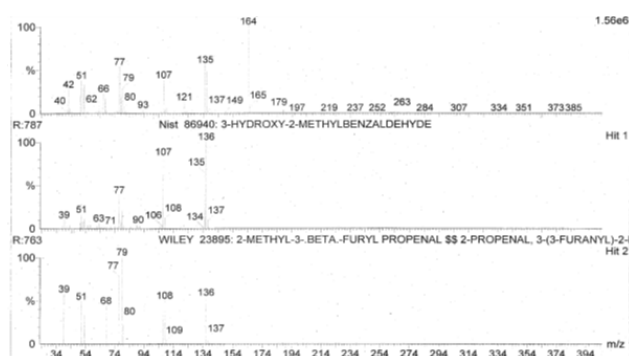


Figure 6: GC-MS analysis.

Table 2: GC-MS analysis.

Hit	REV	For	Compound Name	MW
1	787	551	3-HYDROXY-2-METHYLBENZALDEHYDE	136
2	763	312	2-METHYL-3-.BETA.-FURYL PROPENAL \$\$ 2-PROPENAL,3-(3-FURANYL)-2-METHYL-	136
3	755	328	5-ENDO-5-EXO-(EPOXYMETHANO)-6-METHYLIDENE-7-OXABICYCLO[2.2.1]HEPT-2-E	136
4	751	544	4-HYDROXY-2-METHYLBENZALDEHYDE \$\$ BENZALDEHYDE, 4-HYDROXY-2-METHY	136
5	751	544	4-HYDROXY-2-METHYLBENZALDEHYDE	136
6	745	453	3-HYDROXY-2-METHYLBENZALDEHYDE	136
7	742	326	4-HYDROXY-3-(2-METHYLPROPYL)PYRIDINE \$\$ PYRIDINE, 4-METHYL-3-(2-METHYLP)	149
8	737	509	4-HYDROXY-3-METHYLBENZALDEHYDE	136
9	735	455	3-HYDROXY-4-METHYLBENZALDEHYDE	136
10	729	317	5-EXO,5-ENDO-(EPOXYMETHANO)-6-METHYLIDENE-7-OXABICYCLO[2.2.1]HEPT-2-E	136
11	722	580	2-HYDROXY-5-METHYLISOPHTHALALDEHYDE	164
12	722	496	5-HYDROXY-2-METHYLBENZALDEHYDE	136
13	718	457	BENZO[1,2,5]THIADIAZOLE-4-SULFONIC ACID (ADAMANTAN-1-YLMETHYL)-AMIDE	363
14	715	457	6-HYDROXYCHROMAN-4-ONE	164
15	712	580	2-HYDROXY-5-METHYLISOPHTHALALDEHYDE	164
16	711	375	ETHANONE,2-ETHOXY-1,2-DIPHENYL-(CAS) \$\$ 2-ETHOXYBENZOIN \$\$ BENZOYL E	240
17	711	512	3,4-(METHYLENEDIOXY)TOLUENE	136
18	711	376	ETHANONE, 2-ETHOXY-1,2-DIPHENYL-	240
19	707	429	2-HYDROXY-3-METHYLBENZALDEHYDE	136
20	707	429	2-HYDROXY-3-METHYLBENZALDEHYDE	136

and T3SS.^[17] It is reported that *Pantoea* sp. is found to be responsible for 10 – 15% loss in yield of cotton in the south-eastern US Cotton Belt. Pathogenic bacteria could be transmitted into cotton bolls by the southern green stink bug in the fields of the northern states of India, including Uttar Pradesh, Haryana and Punjab exhibits a appearance of new blight disease on basmati rice (*Oryza sativa* L.). Yellow-pigmented bacteria were identified as a *Pantoea ananatis* responsible for blighted appearance.^[17] Strain of *Streptomyces rochei* has been known for the biological activity against phytopathogenic fungi on Seedlings of sorghum and tomato but it was not investigated properly for metabolite production's point of view in other plant properly.^[18]

Molecular characterizations of metabolites produced by *Streptomyces rochei* were identified as α -Hydroxy-2-Methylbenzaldehyde, 2-Methyl-3-Beta-Furyl Propenal, 6-Hydroxychroman-4-one and 2-Hydroxy-5-Methylisophthalaldehyde. It is reported the presence of 'chromone' nucleus in the compounds extracted from *Streptomyces levis* through structural confirmation by ¹HNMR.^[19]

CONCLUSION

The present study proved that the leaf blight of cotton can also be caused by *Pantoea* sp. To our knowledge this is the very first report of new bacterial leaf blight of cotton caused by *Pantoea* sp. in Mehsana District, India. Molecular characterization of the compound produced by *Streptomyces rochei* revealed presence of Antimicrobial compounds and shows potent activity against *Pantoea* sp.

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CONFLICT OF INTEREST

The authors declared that they have no conflicts of interest.

ABBREVIATIONS

SCA: Starch casein agar; **Sp.:** species; **DW:** Distilled Water; **Rpm:** rotation per minute; **ML:** milli litre; **UI:**

micro litre; **H:** hour/s; **FTIR:** Fourier Transform infrared; **GC-MS:** Gas Chromatography-mass Spectrometry; **NIST:** National institute of standard and technology; **N-agar:** Nutrient agar.

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