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ORIGINAL ARTICLE

Isolation and Screening of antifungal metabolite producing *Streptomyces* sp. from the Soils of North Gujarat, India

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ABSTRACT
Twenty-five strains of Streptomyces sp. were isolated from cultivated soils of Mehsana District in North Gujarat, India (latitude:23.58, longitude:72.36, elevation: 272/ 83ft/m).All the isolates were belonging to Streptomyces genera. The isolates were checked for antifungal activity against plant pathogenic fungi infecting cotton (Gossipium hirsutum) balls. The mycelia of one selected isolate Gray-1 out of all isolates, was extracted using ethyl acetate and tested against infectious fungi.After primary and secondary screening, isolate Gray-1 showed strong antifungal activity. Morphological, Physiological characteristics and 16S rRNA sequence homology studies had shown that the isolated strain of Streptomyces sp. was similar to Streptomyces rochei (sequence similarity 99.43%, GeneBank accession number MN114054). The infected fungus was identified as an Aspergillus ruber through genetic analysis (Gene Bank accession number (MN133921).This is the first report of antifungal activity of Streptomyces rochei against pathogenic fungi of cotton balls.It can be used for the improvement of new substances for agronomic aspects.
Keywords: *Streptomyces sp., Antifungal metabolite, Plant pathogen, Gossipium hirsutum*

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INTRODUCTION

Most of the antibiotics commercially available are produced by bacteria, fungi, plants and actinomycetes. Among the various genus of actinomycetes, *streptomyces* sp. have the capacity to produce variety of metabolites [1,22]. The actinomycetes are differentiated from other bacteria based on their morphology, G+C content and other genomic features. They contain high G+C content (>55%) in their DNA [2] as compared to other species.

Commercially important metabolites are commonly produced by *Streptomyces, Saccharopolyspora, Actinoplanes, Micromonospora, and Amycolatopsis*. About, 80-85% of widely known antibiotics have been produced by using *streptomyces* sp. Due to their characteristics like utilizing organic matter, high metabolicrate of production and capability to degrade lignocelluloses, chitin, etc. more importance is given to them in order to isolate novel species for antibiotic production [3]. Selection of an un-investigated soil samples is done in order to isolate streptomycessp. which may give novel bioactive metabolites.

Various fungal diseases along with virus, nematodes, bacteria and pests may effect on yield of cotton plant [4]. Fungi are found to be important plant pathogens of various plants like Cotton, Lemon, Tobacco, Castor etc. *Aspergillus* sp. and other fungi are the most common pathogens which infect cotton balls and detoriates the quality of cotton fibres [5].

The current study involves the isolation of *Aspergillus* sp. associated with cotton balls and isolation of Streptomyces strains from agricultural soils of North Gujarat with prospective to produce active

antifungal compound exclusively against *Aspergillus*sp. Additionally, the isolated strains of actinomycete was characterized and recognized based on physiological, morphological, biochemical, and genomic characteristics.

MATERIAL AND METHODS

Study area and collection of soil samples

The study field was located at various districts of North Gujarat; India. Total 20 sites were randomly selected for the study. The samples were collected from the duration of April 2018 to July 2018 from the field.The soil samples from the rhizospheric areas of cultivated plants like Cotton, lemon and castor were collected from different sites in North Gujarat. These samples were transferred to the laboratory in sterilized dried polyethylene containers following standard measures and kept at storage temperature until further use so that it couldn't get contaminated.

Isolation of Streptomyces sp.

After collection, one gram of soil sample was added to 100 mL of sterile water, mixed well and then placed on rotary shaker at 150 rpm for 1hr. 1 ml of each serially diluted sample (10^{-1}, 10^{-2}...10^{-10}) was added in to 50 mL of SCA molten agar medium. The antibiotic Rifampicin (25 µg/mL) was supplemented in the medium to prevent bacterial contamination. The medium was poured in sterile Petri plates. All the plates were incubated at 30°C and the growth of Streptomyces sp. were observed during incubation time to time [6]. After 10 days of incubation, pure colonies of Streptomyces sp. having chalky, rough, powdery, leathery and texture appearance were selected and preserved on Streptomyces sp. isolation agar medium (HiMedia) at 30°C for 48hrs.

Collection of infected samples and isolation of fungal pathogens

The infectious cotton balls were collected from local farming area of Mehsana District and used for the isolation of fungal pathogens. All the infected cotton balls were stored at 4°C in sterile polythene bags. The fungal cultures from infected cotton balls were isolated on potato dextrose agar medium (Hi Media) [7]. The diseased sample was mixed in a test tube containing 10 mL of sterile distilled water. 1 mL of 10^{-6} diluted suspension was spreaded on potato dextrose agar plates and further incubated at 30°C for 72 h.

Primary Screening of Streptomyces sp. for antifungal metabolite Production

The antifungal activities of the actinobacterial isolates against fungal pathogens were evaluated by using cross streak-plate method[8]. Single streak of the active culture of actinobacteria was prepared on the surface of Starch Casein Agar medium, and incubated at 28±2°C. After growth of actinobacteria as a good ribbon like pattern at the centre of the plates, the suspension of fungal pathogens was streaked at right and left angles to the original streak of actinobacteria and incubated at 37°C for 72 h. The selected actinobacteria with antifungal activity in preliminary screening were further tested for secondary screening by well diffusion assay.

Secondary screening of the isolate Gray-1

The strains of Streptomyces sp. Gray-1 show potent antagonistic activity against *Aspergillus* sp. in primary screening and was tested further for bioactive metabolite production. The active culture of isolate was inoculated into Starch casein broth for the production of bioactive metabolite (150 rpm) at 28 °C for 7 days. After incubation, the active broth was separated by centrifugation (6000 rpm/20 min) and supernatant was used for testing antimicrobial activity. The antimicrobial activity was tested from crude supernatant by agar well diffusion method against *Aspergillus* sp. The activities of isolated Streptomyces sp. were estimated by adding 50 µL of the supernatant to Mueller Hinton agar medium inoculated with 50µL of culture of test organism. All the plates were placed at 8°C for 15 min to allow diffusion of antibiotic and then incubated at 25°C for 48 h. The diameters of zone of inhibition surrounding the wells were measured and all the experiments were performed in triplicate.

Fermentation of bioactive metabolites

The fermentation media was prepared by adding following ingredients (per litre of distilled water): 10 g starch, 4 g yeast extract, 2 g peptone, 5 ml potassium bromide (20 g/L), 5 mL iron and sulphate tetrahydrate (4.76 g/L). The medium was transferred into 500 mL amount into 1 L Erlenmeyer flasks and sterilized by autoclaving at 121 °C for 15 minutes. After cooling of the medium, 100 µL of Streptomyces sp. culture (as per McFarland 0.5) were used to inoculate the flasks. The flasks were then incubated at 30 °C on a rotary shaker at 150 rpm for 12 days. The purity of cultures was confirmed by streaking the fermentation cultures onto potato dextrose agar (PDA), nutrient agar (NA), and starch casein agar (SCA) plates.

Characterization of Actinomycete (Gray-1)

The potent actinomycete (Gray-1) was determined by morphological, biochemical and physiological characteristics according to International Streptomyces Project [9].The colony characteristics such as

colony texture, colour of substrate, aerial mycelia, colony reverse colour and growth pattern were recorded growing on starch casein agar medium [10].

Morphological characterization

The arrangements of spores, substrate and aerial mycelium, spore bearing hyphae of isolated Streptomyces sp. were observed by using a Binocular microscope (Olympus) under 400X magnification.

Physiological and biochemical characterization of isolated actinomycete

The ability of isolate to grow at different temperatures, pH and NaCl tolerance were tested. The carbohydrate utilization by using several carbon sources such as glucose, fructose, galactose, mannitol, sucrose, xylose, raffinose, lactose, and sorbitol were tested. Biochemical characterization such as carbohydrate fermentation test, nitrate reduction, hydrolysis of casein, hydrolysis of starch, citrate utilization, methyl red, voges-proskauer, indole, urea, oxidase, catalase, were tested for isolate Gray-1.

Genetic characterization of isolated actinomycete

The isolate Gray-1 was characterized by 16S rRNA gene sequencing and phylogenetic tree construction was carried out. The Alignment tool (BLAST) was used to check the similarity between the available sequences from database.

Extraction of Crude Extracts from Fermentation Culture

Solvent extraction using ethyl acetate was used to extract crude metabolites from the broth culture filtrate as per description of Liu, C. *et al.,* 1996. Equal volume of Ethyl acetate was added to the filtrate and kept under vigorous shaking for 1 h to extract crude product. The solvent phase that 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. Test organism cultures were grown overnight (48 h) in Potato dextrose broth. 1 ml of Test organisms were added in 20 ml of Muller Hinton molten agar tubes and poured onto Muller Hinton agar plates. A diameter of 6 mm was prepared by sterilized cork borer to bore wells into the agar and 50µL of the extract loaded into the wells. The extract was allowed to diffuse into the agar before the plates were incubated under aerobic conditions at 37 °C for 24 h. The wells loaded with Ketoconazole (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.

RESULTS

Isolation and screening of Streptomyces sp. for antifugal metabolites

The currentinvestigation involves the screening of novel antibiotic producing Streptomyces sp. from soils of Mehsana District, North Gujarat. Total twenty-fivestrains of Streptomyces sp. wereevaluated for primary and secondary screening against infected fungi. Among them isolate Gray-1 shows potent activity against pathogenic fungi infecting the cotton balls (Fig. 1 and Table 1). The isolated fungus from infected cotton balls was identified as an *Aspergillus ruber.* The isolate Gray-1 was sub-cultured on Starch casein agar medium and preserved in refrigerator for further studies.

Table 1 Antifungal activity of Gray-1 by well diffusion assay

Sr. no.	Sample code	Zone diameter in (mm) against *Aspergillus ruber*
1	Crude extract of Gray-1	21
2	Ketoconazole (50 µg/mL)	29

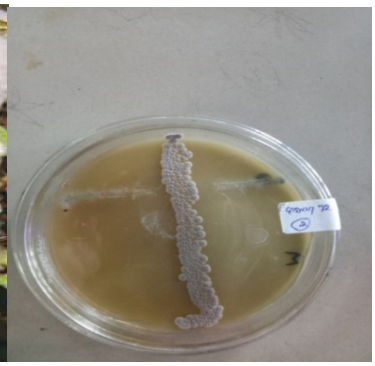

Fig.1 Cotton balls infected with *Aspergillus* sp. **Fig.2** Primary screening of antibiotic production by Gray-1

Secondary screening

In primary screening, about 15 Streptomyces sp. strains were selected based on their efficiency. In the secondary screening, 14 actinomycete isolates shows activity in well diffusion assay. However, the crude extract of the actinomycete isolate Gray-1 exhibited prominent activity with large area of zone of inhibition against the isolated fungi. Crude extract from isolate Gray-1shows 21 mm Zone of inhibition against *Aspergillus ruber* (Table 1).

Morphological and physiological Characterization of isolate Gray-1

Based on physiological, biochemical and molecular characteristics the isolated strain was aerobic and non-motile Gram positive in nature. The colony diameter is 1 to 2 cm in range and the colour of the colony was greyish on upper side and yellowish on the reverse side. The isolate was observed to grow best in mesophilic temperature range, exhibited catalase, amylase, and protease activities. It didn't produce oxidase, indole and hydrogen sulphide and was found to be negative for urea reaction, citrate utilization, methyl red test, nitrate reduction and voges-proskauer test.

(Table 2). The isolate Gray-1 utilized the carbon sources such as arabinose, fructose and sucrose. The organism did not utilize xylose, raffinose and sorbitol. The isolate was able to grow up to 40°C with pH 8 and 6% NaCl, the optimum growth temperature was found to be 28 ± 2 °C (Table 3).

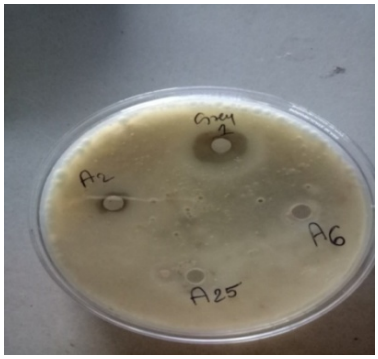

Fig.3 Secondary screening of isolate Gray-1

Table 2 Physiological and Biochemical characterization of isolate Gray-1

Characteristics	Data for isolate
Gram staining	+
Reduction of nitrate	-
H_2S Production	-
Oxidase	-
Casein Hydrolysis	+
Starch Hydrolysis	+
Lipid Hydrolysis	+
Citrate utilization	-
Voges-Proskauer test	-
Methyl red test	-
Indole production	-

Table 3: Results of Carbon source utilization and tolerance to NaCl, temperature and pH by Gray-1

Characteristics	Data for isolate
Utilization of carbon sources	
Sucrose	+
Sorbitol	-
Fructose	+
Galactose	+
Glucose	+
Xylose	-
Lactose	+
Raffinose	-
Maltose	+
Mannitol	+
NaCl tolerance	

1%	+
2%	+
3%	+
4%	+
5%	+
6%	+
7%	-
pH	
pH 5	-
pH 6	+
pH 7	+
pH 8	+
pH 9	-
Temperature	
10 °C	-
15 °C	+
20 °C- 45 °C	+
50 °C	-

Genetic characterization of isolate Gray-1

16S rRNA sequencing of isolated Streptomyces sp.strain was identified as *Streptomyces rochei*(Fig.4**).** The phylogenetic treewas constructedwith the aid of MEGAX(Molecular Evolutionary Genetic Analysis X) software using neighbour-joining method for sequence alignment and matching.In the process, statistical method of bootstrapping was used for constructing the consensus tree groups and distances on side chains.The isolate revealed relationship with a group of Streptomyces species (Fig.5) [11].

GGCTCTAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGC
GAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGG
CGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGT
AGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACG
CTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGT
TGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG
GCGGAGCATGTGGGCTTATTTGACGCAACGCGGAAGAACCTTACCAAGGCTTGACATACACCGGAAACCCTGGAGACAGGGTCCCCCTTGTGGT
CGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAG
CAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCT
TGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGT
TCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGA

Fig.4 Aligned Sequence Data of GRAY-1 (1060 bp)

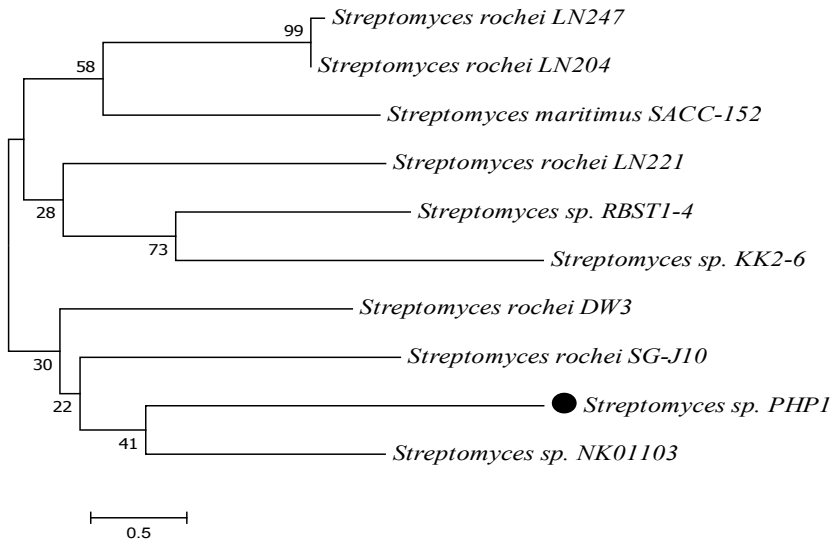

Fig.5 Phylogenetic tree of Gray-1

DISCUSSION

Various plants having agronomic value are threatened by fungal infections and effects on the quality of various plants having economic value. There is a need for development of new class of antifungal drugs due to limited availability of antifungal metabolites in agriculture and development of rapid drug resistance, [12].The fungi which infects on cotton plants majorly found to be as *Aspergillus flavus ,Alternaria alternata,*., *Ascochyta*sp., *Arthriniums*p*., Aspergillus niger, Aspergillus* sp., *Cercospora*sp., *Botryodiplodia*sp., *Chaetomium* sp., *Cladosporium* sp., *Chaetophoma*sp., *ColletotrichunCoffeanum*., *Colletotrichum* sp., *Curvularialunata*, *Curvularia clavate Fusarium moniliforme*, *Fusarium* sp., *Nigrospora*sp., *Pestalotia*sp., *Trichoderma viride., Penicillium* sp., *Phoma* sp., *Pteroconium* sp., *Puccinia* sp., *Rhizopus stolonifer* and *Sclerotiumrolfsii* [4].

Antimicrobial effects of actinomycetes have long been widely studied against animal pathogens; but their antimicrobial activity against plant pathogens has not gone to that extent and there is a huge gap in its understanding. Hence, searching for novel antibiotics from soil of unexplored region is the main aim of this effort.The isolated strain can be used as effective antimicrobial agent against fungus of cotton balls. Extract of the isolated strain is found to be as effective as ketoconazole and can be used as a substitute of that being nontoxic to plants in nature.

Aspergillus ruber is a target pathogen which causes infection on cotton balls. Isolate Gray-1 was found to be a good antifungal producer against *Aspergillus ruber*. In well diffusion assay crude supernatant of antibiotic extract shows good zone of clearance.

*Streptomyces rochei*is able to grow at up to pH 8 and 6% NaCl solution. The isolate is able to grow in mesophilic temperature range and having excellent potential to produce antifungal metabolites. The crude antifungal metabolite produced by the *Streptomyces rochei* is found to be thermostable and it also shows antifungal activity in wide range of pH (2–8).

ketoconazole (10mg/ml) showed 10.23 mm of zone of inhibition against *Aspergillus* sp. [13]. Effect of ketoconazole (50mg/ml) against *Aspergillus ruber*was seen as inhibition zone of 29 mm and the crude antibiotic extracts made the zone of 21 mm, which is quite satisfactory in results.

Streptomyces rocheiACTA1551 has been found to be most effective to protect tomato seeds from *F. oxysporum* infect on tomato plants [14]. Fungus also have ability to produce some hydrolytic enzymes like chitinases, proteases, or β-1,3glucanase that cause degradation of fungal cell wall [15, 16, 17].

The crude supernatant was extracted with various solvents. It isresistant against high temperature. It has been reported that *Streptomyces violaceusniger* YCDE9 [18, 22] and *Streptomyces hygroscopicus* [19] have the ability to produce hydrolytic enzymes during exponential phase and antifungal metabolites during stationary phases. The antifungal activity of *Streptomyces rochei*is highly polar in nature and produced during stationary phase.

Kaur and co-authors have mainly focused on isolation of secondary metabolites producing actinomycetes strain from soil from (Dalhousie, 32.53°N, 75.98° Himachal Pradesh, India) and investigation of its effectiveness against fungal Phyto-pathogens [15, 20]. Antifungal activity of terrestrial*Streptomyces rochei* strain HF391 against clinical azole -resistant *Aspergillus fumigatus* was also investigated*[21].* Biocontrol ability is attributed to its strong production of antibiotics like antifungal nigericin, and antibiotic geldanamycin from S. violaceusniger YCED-9 [22].Antagonistic potential of *Streptomyces flavomacrosporus* GACMPT-showed inhibitory activity against the plant pathogens, *Rhizoctonia solani* and *Fusarium oxysporum* in Indian soil[23]. Wide-ranging studies on antifungal activity of novel secondary metabolites and their characterization were not carried out till date. In contrast, our current research demonstrates a discrete line of objective in terms of source, isolation, applications, mechanism of action and stability proving its novelty and uniqueness**.**

CONCLUSIONS

As mentioned in results and discussion, isolate Gray-1 *(Streptomyces rochei)*can be further tried on the fields against fungal pathogen of cotton balls and can be commercialized as other means of solving fungal pathogenic problems as other means are either not so effective or harmful to humans and other animals. Further investigations are necessary for molecular characterization of the compound produced by *Streptomyces rochei* through chromatographic and other spectral analysis which can explain more about its future usability.

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CONFLICT OF INTEREST
The authors declared that they have no conflicts of interest.

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