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Isolation and identification of *Streptomyces* sp. producing agroactive enzymes with biocontrol potential

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Abstract

The enzyme chitinase has potential application in the field of agriculture. The aim of the present study is the isolation, characterization and identification of the potential chitinase-producing bacteria from the Nagari farm tea garden of Darjeeling Hills. Around twelve actinobacterial strains were isolated and screened for their chitinase and cellulase activities followed by the antagonistic activity against fungal pathogen *Fusarium* sp. The potential isolate with antagonistic activity was then characterized and identified as *Streptomyces* sp. SDr 22. The isolate tolerated the NaCl concentration of 1% to 4% and could grow in the pH range of 5 to10 at an optimum temperature of 30ºC. The presence of chitinase-producing *Streptomyces* isolate in the tea garden of Darjeeling Hills with biocontrol activity has been explored in this study.

There is a great demand for agricultural land for the cultivation of crops to supply food to the ever-increasing world population. However, there are limitations of the land that can be cultivated which increases the immediate need to control crop diseases and increase the yield with the limited land resource. Both the abiotic and biotic factors are responsible for the productivity of crop plants. One of the major factors causing a decrease in the yield of crops is fungal plant pathogens.[12] The pathogens causing various diseases to plants are being controlled by various chemical insecticides and fungicides. However, these chemicals are also causing harm to the environment thereby affecting both animal and human health.[33] Global warming accompanied by climatic change has increased the incidence of various fungal diseases and causing damage to crop plants. This has posed a great loss in the agricultural sector and production of food.[2]

Chitin is present in the exoskeleton of arthropods such as crustaceans and insects and is the most abundant biopolymer in nature

after cellulose.[33] It is also one of the components of the cell wall of fungal pathogens. Chitinases play a role of importance in the degradation of chitinous waste.[35] Chitinases are glycosyl hydrolases that hydrolyze the chitin in the insoluble form to pharmaceutically valuable products such as chitooligosaccharides and glucosamines which are soluble forms. These chitooligosaccharides are known to have antifungal, antibacterial, antitumor and immune-enhancing effects. The bacteria producing chitinases with biocontrol potential against pathogenic fungi have been exploited.[17] Thus the role of chitinase producing microbes as biocontrol agents cannot be ignored in such a scenario.

Actinobacteria is one of the main sources of bioactive secondary metabolites and commercially important enzymes in both the medical and agricultural fields.[16] They play important role in the cycling of carbon sources such as chitin and cellulose.[14] Actinobacteria especially from the genus *Streptomyces* which are are filamentous, gram-positive, long rods have chitinolytic activity. Several *Streptomyces* sp such as *S. griseus, S. lividans, S. plicatus, S.aureofaciens, S. halstedii, S.glauciniger* are known for producing chitinolytic enzymes.[4,16,30] They have antagonistic activity towards many pathogenic fungal species. *Streptomyces* is known to control Fusarium wilt.[37] The considerable affinity of chitinase to chitin has lead several biotech companies to explore the potential for the development of disease-resistant seeds and transgenic plants.[12]

In the present study, we isolated the actinobacterial strains from Nagari farm tea garden soil. The strains recovered were screened for the production of the enzymes cellulase and chitinase. The potential enzyme producer selected and studied further for their antifungal activities against the phytopathogen *Fusarium* sp. This isolate with the good antagonistic activity was characterized morphologically, biochemically and physiologically and identified by 16s rDNA sequencing.

Isolation :

Tea rhizospheric soil samples were collected from Nagari farm tea garden located in Darjeeling hills, West Bengal, India. The soil samples collected were air-dried for five days at 30ºC. The soil sample dried and was serially diluted in saline water containing 0.85% NaCl.[31] The inoculation was done from each dilution by the method of spread plate. For isolation, the media used for growth were Starch nitrate agar[11] and Inorganic Salts, Starch Agar (ISP4).[31] The inoculated plates were incubated at 30°C for seven days. After purification, the isolates were sub-cultured using ISP4 slants and stored at 4°C. [24].

Screening for the production of chitinase and cellulase :

Chitinase assay :

The isolated strains inoculated into chitin agar media (Colloidal chitin 5g; KH_2PO_4 0.03g; K_2HPO_4 0.07g; $FeSO_4.7H_2O$ 0.001g; $ZnSO_4.4H_2O$ 0.0001g; $MgSO_4.7H2O$ 0.05g; $MnCl_2.4H2O$ 0.0001g; agar 2g; ddH_2O 100 ml, pH adjusted to 7.0). Colloidal chitin was prepared[7] and the plates after inoculation were incubated for 5 days and the plate flooded with gram's iodine solution and the halo around the colonies were recorded.[22]

Cellulase production :

The isolated strains were inoculated into CMC Agar (Carboxy-methylcellulose 0.5g, $K_2HPO_4$0.1 g, $NaNO_3$ 0.1 g, yeast extract 0.05 g, $MgSO_4$0.05 g, Agar15 g, distilled water 1000 ml) plates and incubated at 30°C for 5 days. After completion of incubation, plates were flooded with Gram's iodine solution which resulted in the formation of a bluish-black complex with unhydrolyzed cellulose. The clearance zone around the colonies is the indicator of a positive result.[10]

Antagonistic activity against fungal pathogens:

The strains under study were tested in vitro for their antagonistic activity against fungal root pathogen, *Fusarium solani* (RHS/P388) with accession number NAIMCCF-02901 and other *Fusarium* species obtained from immunopathology Laboratory, Department of Botany, North Bengal University. The antagonism test was carried out in Potato Dextrose Agar (PDA) by the method of dual culture[36] with slight modification. Freshly grown *Fusarium* sp. inoculated on one edge of the plate and the isolated strain SDr 22 inoculated in the same plate at the extreme opposite edge of the plates by streaking, sealed tightly with parafilm. The control plate was only inoculated with *Fusarium* sp. After inoculation plates were incubated for 10 days at 30°C.

Morphological studies :

Morphological studies were done by growing the isolates at 30°C for 7 days in various International Streptomyces Project (ISP) Medium such as Tryptone-yeast extract broth[19], Yeast extract-malt extract agar,[20] Oatmeal agar,[13] Glycerol-asparagine agar,[21] Inorganic salts-starch agar[13] Peptone-yeast extract iron agar,[32] Tyrosine agar.[25] The formation of the substrate and aerial mycelium was studied.[26]

Biochemical and Physiological Studies :

The biochemical tests that were carried out were the formation of Melanin,[26] degradation of Casein Tyrosine, Xanthine,[5] hydrolysis of Esculin[9], production of Amylase,[8] Gelatinase,[27] Lipase,[3] reduction of Nitrate,[15] and Carbohydrate utilization test.[26] Growth of the isolate in various pH(5-10),NaCl concentration (1% to 7%) and various temperature (25°C,30°C,40°C) evaluated using Bennet Agar media.[28]

DNA isolation and 16srDNA sequencing :

The isolation and purification of DNA of the potential isolate were carried out following CTAB method.[34] The amplification of 16S rDNA sequence carried out by Polymerase Chain Reaction (PCR) using primers ACT235F (5'CGC GGC CTA TCA GCT TGT TG3') and ACT 878R (3'CCG TAC TCC CCA GGC GGG G5'.[29]

Isolation and Screening for the production of chitinase and cellulase :

Colonies of actinobacteria were found to be white and chalky in the isolation media used namely Inorganic Salts Agar medium and Starch Nitrate agar medium. Total twelve

colonies of actinobacteria were recovered and they were found to be a producer of chitinase and cellulase. The isolate SDr 22 with the most potential ability to produce cellulase (Fig 1a) and chitinase (Fig 1b) were also found to exhibit good antifungal activity against plant pathogenic fungus *Fusarium* sp (Fig 1c). The control plate was fully grown by the *Fusarium* sp. Whereas in the plate with both *Fusarium* sp and SDr 22 inoculated, the growth and spread of *Fusarium* sp. in the entire plate was suppressed.

Morphological, biochemical, physiological characterization and 16s rDNA sequencimg:

Morphological studies of the isolate showed the strain showed variation in the coloration of Aerial and Substrate mycelium in all the tested media with variation of spore mass coloration (Table-1).

In biochemical studies it was found that the isolate produced amylase, cellulase and lipase and utilized tyrosine and esculin however failed to produce gelatinase, reduce nitrogen and hydrolyze casein. In carbohydrate utilization studies it was found the isolate SDr 22 utilized all the tested sugars namely glucose, arabinose, fructose, sucrose, xylose, inositol, rhamnose and raffinose. The isolate tolerated NaCl concentration of 1% to 4% and showed growth in the pH range of 5 to 8.

The Molecular identification of the strain was done by Sanger sequencing of 16S rDNA gene fragment after amplification by PCR and the phylogenetic tree constructed by the neighbor-joining method with the sequence obtained from *Streptomyces* sp SDr 22 and other *Streptomyces* sp.(Fig 2).

The similarity of the sequence studied through the online NCBI BLAST program, where it was found the isolate SDr 22 showed 99.50 % similarity to *Streptomyces* sp. By the similarity-based searches in the ezBioCloud server the isolate showed a similarity of 97.25% to *Streptomyces flavovirens.* The 16S rDNA gene sequences were submitted to NCBI and the accession number provided was MK300088.1.

A good percentage of the soil microbial community comprises actinobacteria with *Streptomyces* being the major genus known for producing antibiotics, bioactive compounds and various extracellular enzymes of importance. This genus has huge potential to improve agricultural productivity in the future.[18] Our study supports the findings where the *Streptomyces and* non-*Streptomyces* species producer of hydrolytic enzymes such as cellulase, chitinase, protease, and pectinase could degrade fungal and bacterial cell walls thus causing antagonism against fungal pathogens.[6] The *Streptomyces* sp. with chitinolytic activity plays the role of importance in the biocontrol of plant diseases caused by *Fusarium* sp. and attention is also being given to identifying the bioactive metabolites that are accountable for biocontrol. *Streptomyces* is the major microbe responsible for the suppression of diseases in plants.[23] Plant growth-promoting rhizobacteria including *Streptomyces,* with antagonistic activity against *Fusarium* sp, induces the immune system of the plant against various biotic stresses and thus are the better alternative to chemical pesticides and fungicides in the agricultural system.[1]

Table-1. Morphological characterization of SDr 22 in various ISP media

Medias	Aerial	Substrate mycelium mycelium	Spore Mass	growth
Tryptone Yeast Extract Agar	Off White	Off White	No Spore Mass	++
Yeast Malt Agar	Off White	Off white to Brown	Whitish	+++
Oatmeal Agar	Off White	Brownish Grayish	Brownish	+++
Inorganic Salt Starch Agar	White	Greenish	Olive Green	+++
Glycerol Asparagine Agar	Off White	Off White	Off White	++
Peptone Yeast Extract Iron Agar	Off White	Off white to Light Brown	Off White	+++
Tyrosine Agar	Off White	Grayish Brownish	Grayish	+++

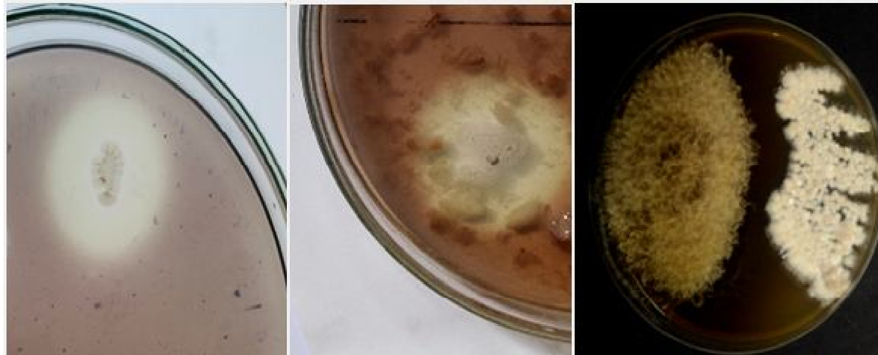

Fig1 (a). Cellulase production by strain SDr 22 in CMC agar after flooding with iodine solution (b). Chitinase production in Colloidal chitin agar by strain SDr 22, with a zone of clearance around colony after flooding with iodine solution. (c). Antagonistic activity of SDr 22 against *Fusarium* sp. after 10 days of incubation.

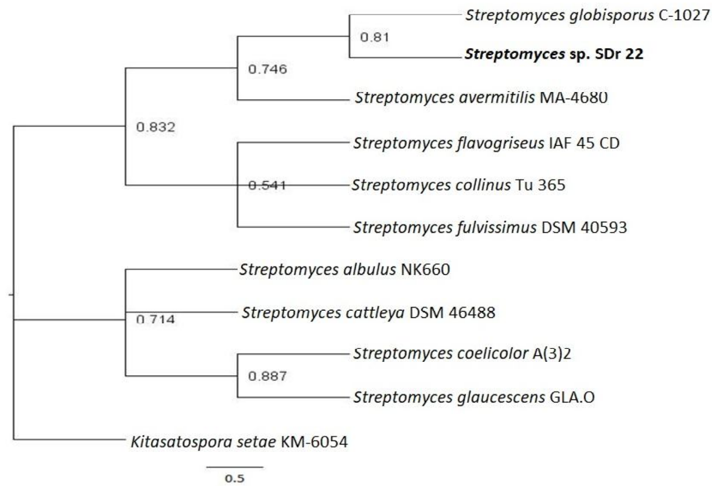

Fig 2: Phylogenetic tree based on the partial 16s rRNA sequences

In this study, the actinobacterial strains from tea garden soil were assessed for the production of cellulase and chitinase. The potential enzyme-producing isolate SDr 22, with good antifungal activity, was found to belong to genus *Streptomyces* based on morphological, biochemical and 16srDNA sequencing studies. Thus *Streptomyces* sp. SDr 22 represents the indigenous tea rhizosphere population of tea rhizospheric soil and can be explored for the preparation of biofertilizer in future. Chemical pesticides and fungicides are being used for many years however the use of eco-friendly alternatives will be a boon to the ecosystem thereby preventing the damage further.

Conflict of Interest :

The authors declare no conflict of interest.

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