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Anti-fungal potentials of extracellular metabolites of Western Ghats isolated *Streptomyces* sp. NII 1006 against moulds and yeasts

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Realization of hazardious effects of chemical fungicides has led to an interest in the usage of biocontrol agents. The present study, therefore, evaluates the biocontrol efficacy of Western Ghats (India) soil bacterial isolates. A potential strain NII 1006 was evaluated for its antagonistic property against a diverse range of moulds and yeasts. The strain was characterized morphologically, biochemically and molecularly, which revealed the isolate belonged to *Streptomyces* genus. Organic solvent extracts of NII 1006 culture filtrates inhibited the growth of the test pathogens indicating that growth suppression was due to extracellular anti-fungal metabolites present in the culture filtrates. The strain produced extracellular chitinase enzyme in addition to some stable partially purified anti-fungal compounds. Morphological changes such as hyphae degradation into debris and abnormal shapes were observed in test fungi and yeast grown on potato dextrose broth that contained the NII 1006 culture filtrate. The cell free supernatant has a tolerance to wide range of *p*H, temperature and enzymes such as lipase and protease. The biocontrol potential of NII 1006 strain may be correlated significantly with their ability to produce antibiotics as well as extracellular hydrolytic enzymes particularly chitinolytic enzyme.

Keywords: Anti-fungal activity, Biocontrol, Extracellular anti-fungal metabolites, *Streptomyces*

Fungal phytopathogens pose serious problems by damaging crops world-wide causing heavy economic loss to agriculture and food industry. In modern agricultural era, to meet the day by day increasing demand of food supply, chemical fungicides are being used intensively & exhaustively which may lead to serious issues like health hazards, environmental pollution and development of resistivity of pathogens. One alternative way to maintain the populations of pathogenic fungi at low levels is the usage of biocontrol agents which is gaining interest recently by public concerns over the use of chemical fungicides. Many species of actinomycetes, particularly those belonging to the genus *Streptomyces*, are well known as anti-fungal biocontrol agents that inhibit several plant pathogenic fungi[1-4]. The antagonistic activity of *Streptomyces* sp. against fungal pathogens has been usually related to its efficiency in producing of anti-fungal compounds [5-8] and extracellular hydrolytic enzymes [9-11].

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The rich forests of the Western Ghats in Kerala harbor a large portion of India's biological diversity that include most of the endemic and less explored species. Serious attempt has not been made to explore this rich resource for crop disease management. Hence, the present study was undertaken to isolate and evaluate potent actinomycetes strains that are shown to be effective in inhibiting the growth of fungal phytopathogens thereby facilitating plant disease management.

Materials and Methods

Sampling and isolation of actinomycetes—The strains used in study were isolated from the randomly collected soil samples of terrestrial forest in Silent Valley, Western Ghats of Kerala (11°08′N 76°28′E), India. To enrich the population of actinomycetes, the soil samples were subjected to heat treatment & calcium carbonate treatment [12]. Microbes were isolated by standard spread plate method on ISP2 agar plates by serially diluting the soil samples.

Maintenance of test fungi and yeasts—The test fungi *Botrytis cinerea* (NII08135); *Aspergillus niger* (ATCC98503)*; Rhizopus oryzae* (NRRL1891)*; Oidium*

sp. (NII1201); *Geotrichum candidum* (OKI605/8402); *Fusarium oxysporum* (KACC42109); *Penicillium expansum* (NII08137); *Phytophthora capsici* (NII1202); *Aspergillus flavus* (KACC40244); *Penicillium chrysogenum* (NII08138); *Candida albicans* (MTCC3017) were grown on potato dextrose agar (PDA) plates and incubated at 28 °C for 48 h. Stock cultures were maintained on PDA slants and stored at 4 °C.

In vitro evaluation of antagonistic microorganisms—Antagonistic property of isolated actinomycetes was evaluated by agar well diffusion assay. The isolated cultures were grown in 50 mL of ISP2 broth at 30ºC for 120 h in an incubator shaker followed by centrifugation at 8000 × *g* for 20 minutes at 4ºC and the supernatant was collected. A volume of 50 µl of the supernantant was added to the well bored on PDA plates swabbed with 20 µL of test fungi (10^6 spores/mL suspension) The PDA plates were then incubated at 30 °C for 48 h during which activity was evidenced by the inhibition zone surrounding the well. Each test was performed in triplicates times and the anti-fungal activity was expressed as the mean of inhibition zones (mm) produced by the anti-fungal metabolites when compared to controls.

Cultural and morphological observations of the selected strain NII 1006—Generic identity of the selected isolate was determined by using a procedure that combined morphological and 16S rRNA sequence-based phylogenetic analysis. Cultural and morphological features of NII 1006 were characterized following the directions provided by the International *Streptomyces* Project (ISP) established by Shirling and Gottlieb[13]. Cultural characteristics of pure culture in various media (ISP 1–7, SC agar and TS agar) were recorded after incubation at 28 °C for seven days. Color determination was compared with color chips from the ISCC-NBS COLOR CHARTS standard sample No.2106[14]. For Scanning electron microscopy (SEM) analysis, the stubs containing culture were gold sputtered using SC7620 Sputtercoater device and analyzed by SEM (Zeiss Evo-18 Special Edition).

Molecular analysis of strain 1006—Genomic DNA was isolated as described by Hopwood *et al* [15]. The 16S rRNA gene of NII 1006 was amplified by polymerase chain reaction, using universal primers, 1492R (5′-TACCTTGTTACGACTT-3′) and 27F (5′-AGAGTTTGATCTGGCTCAG-3′. Homology of the 16S rRNA sequence of isolate was analyzed by using BLAST program from GenBank database database (http: www.ncbi.nlm.gov/BLAST/). Multiple alignments with sequences of the most closely related actinomycetes and calculations of levels of sequence similarity were carried out using CLUSTAL_X[16]. A phylogenetic tree was constructed using the neighbor-joining method of Saitou and Nei [17] from K*nuc* values using MEGA version 5.0[18,19]. The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein[20] with 1,000 replicates.

Organic solvent extraction of the anti-fungal compounds—NII 1006 was grown in 1L of ISP2 broth for bioactive extracellular metabolites production. Seven days old fermented broths was centrifuged at 8000 × *g* for 20 min at 4 ºC and filtered through 0.2 µm filters (Millipore, Ireland).The anti-fungal metabolites were extracted with various organic solvents like hexane, ethyl acetate and chloroform in 1:1 (v/v) ratio repeatedly until the solvent remains transparent. Solvent fractions were collected, reduced to dryness using rotavapor and bioassayed. Pure organic solvents and sterile distilled water were used as negative controls. Bioactive compound was partially purified by HPTLC using preparative TLC silica gel 60 F254 (Merck) of thickness 2mm and allowed to develop in a solvent system Di-chloro methane: Acetone (3:1). Each resolved compound was then scratched and eluted with methanol from the bound silica followed by repeated centrifugation. The compound was then checked for anti-fungal activity against the test fungi. The molecular weight of the bioactive compound was estimated by MS technique using Axima CFR^+ Spectrometer in FAB ionisation mode.

Detection of chitinase and β-1, 4-glucanase activity—The presence of extracellular hydrolytic enzymes was also evaluated by performing chitinase and β-1, 4-glucanase enzyme assays. Chitinase activity was quantified by following the release of N-acetylglucosamine during the hydrolysis of colloidal chitin[21]. The reaction mixture, containing 0.5 mL of the culture filtrate, 0.5 mL of 1% colloidal chitin, and 1.0 mL of citrate phosphate buffer (*p*H 5.6), was kept in a water bath at 50 °C for 10 min. Reducing sugars released were estimated by

3,5 dinitrosalicylic acid (DNS) method of Miller[22]. One unit of the enzyme activity was defined as the amount of enzyme which catalyzed the release of 1 µmol of reducing sugar per minute under the assay conditions and expressed as enzyme activity in Uml^{-1}.

β-1, 4-glucanase activity was assayed following the release of free glucose from carboxy methyl cellulose as a substrate. The activity was defined and measured according to the method of Mandels *et al*[23].

Effect of crude extracellular metabolites on the test fungal hyphae and yeast morphology—The effect of crude extracellular metabolites of NII 1006 on the morphology of fungi and yeast was evaluated by SEM. Five mL of 48 h old cultures of *Botrytis cinerea* and *Candida albicans* were incubated with 50% of cell-free culture filtrate for 24 h and analyzed.

Effect of enzymatic treatment and temperature on activity of culture supernatant of NII 1006—The sensitivity of crude culture supernatant to enzymes such as proteinase K and lipase were tested. Proteinase K and lipase (Sigma Aldrich) were dissolved in distilled water at concentration 1 $mgml^{-1}$. One hundred µl of culture supernatant was mixed with 100 µl of enzyme and incubated at 30 °C for 3 h. The residual anti-fungal activity of the mixture was then tested by the agar well diffusion method. To determine the thermal stability of anti-fungal metabolites, 2 mL of culture supernatant was taken in each eppendorf and subjected to a range of temperature (30.0, 60.0, 70.0, 80.0, 90.0 °C for one hour set in water bath and autoclaving at 121 °C for 15 minutes). Samples were then removed, cooled and bioassayed for the residual activity by agar well diffusion assay.

Effect of pH, incubation period, carbon source and nitrogen on bioactivity of NII 1006—To analyze the effect of initial *p*H on biomass and anti-fungal metabolite productivity, 50 mL of media (NaCl 0.5g/L; K_2HPO_4 0.2g/L; $MgSO_4.7H_2O$ 0.01g/L; $FeSO_4.7H_2O$ 0.01g/L; Glucose 0.1%; Yeast extract 0.1%) in each flask with initial *p*H varying from 4-9 (with a unit interval) was inoculated with 2% of pre-inoculum and incubated at 30°C for seven days in a shaker incubator. For determining the optimum incubation period for maximum bioactivity, each flask containing 50 mL of media was inoculated with 2% pre-inoculum, incubated for different time intervals and bioassayed. Effect of various carbon and nitrogen sources on the bioactivity was also investigated. The carbon sources used for the study were glucose, galactose, lactose, xylose, arabinose, sucrose and mannose. The strain was incubated in basal medium (NaCl 0.5g/L; K_2HPO_4 0.2g/L; $MgSO_4.7H_2O$ 0.01g/L; $FeSO_4.7H_2O$ 0.01g/L;) with yeast extract (0.1% w/v) as standard nitrogen source and different carbon sources at a concentration of 0.1% (w/v). Similarly, to determine the impact of different nitrogen sources, 0.1% (w/v) of the each nitrogen source was added to the basal medium containing Glucose (0.1% w/v) as standard carbon source. The organic nitrogen sources used were peptone, yeast extract, tryptone, beef extract and yeast extract + beef extract (1:1). For bioassay, *Oidium* sp. was taken as representative test fungi in all the independent experiments.

Statistical analysis—All the experiments were carried out in triplicates and the mean and SD of the inhibition levels were calculated. Data were subjected to one way ANOVA and the significance of differences between means were calculated by Duncan's multiple range test using SPSS for Windows, standard version 16 (SPSS, Inc.), and significance was accepted at $P \leq 0{\cdot}05$.

Results

Isolation of microbes and evaluation of in vitro anti-fungal activity—We intended to make a collection of antagonistic microorganisms from Western Ghats soil samples and to develop potential bioactive molecules for the management of devastating fungal crop diseases. Out of 150 actinomycetes strains isolated, ten strains exhibited biocontrol activity against all the test fungi used. Further, the strain NII 1006 was selected based on multiple inhibition activity and studied in detail. *In vitro* inhibition of test fungi by NII 1006 has been shown in Fig. 1 and Table 1.

Identification based on cultural, morphological and molecular features—The morphological observations of the culture were made in various agar media as mentioned in Table 2. As the strain grew in all tested media except ISP5 with varying in the color of substrate and aerial mycelium. Spores were formed in media tested except SC agar and TS agar. Mycelial morphology was analyzed under SEM revealed presence of mat like structure of mycelia bearing spore chains (data not shown). For molecular characterization, the 16S rRNA gene was partially sequenced (900 bp) and the sequence deposited in Genbank with an accession No. HM036672. The sequence showed

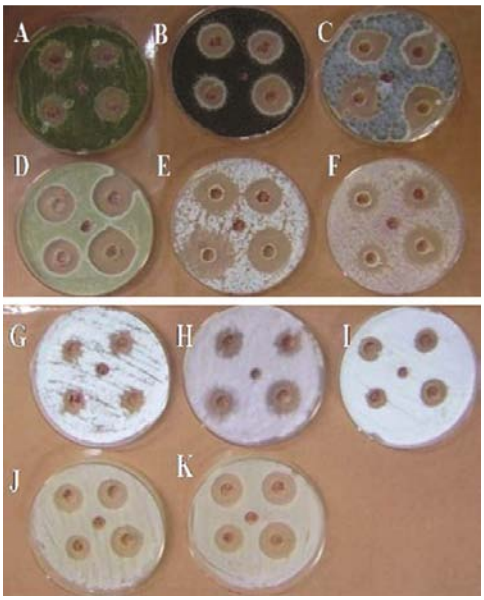

Fig. 1—Inhibition of a wide range of test fungi by NII 1006 culture supernatant in Agar well diffusion assay. A- *B.cinerea*; B- *A.niger;* C- *R. oryzae;* D- *Oidium* sp.; E- *G. candidum*; F- *F.oxysporum*; G- *P.expansum*; H-*P.capsici*; I- *A. flavus*; J- *P.chrysogenum*; K- *C.albicans*

Table 1—*In vitro* inhibition of different test fungi by NII 1006 culture supernatant

Test fungi	ZOI (mm)[a]
Penicillium chrysogenum NII08138	20±1.0
Candida albicans MTCC3017	25±0.5
Aspergillus niger ATCC98503	14±0.0
Oidium sp. NII1201	34±0.5
Rhizopus oryzae NRRL1891	16±0.5
Penicillium expansam NII08137	18±0.5
Geotrichum candidum OKI605/8402	25±0.5
Fusarium oxysporum KACC42109	12±0.5
Aspergillus flavus KACC40244	12±0.5
Botrytis cinerea NII 08135	15±0.5
Phytophthora capsici NII1202	18±0.0

[a] Standard deviation (SD) calculated (n=3)

99.8% similarity to *Streptomyces violascens* and forms the monophyletic clad which is supported by 100% bootstrap values (Fig. 2).

Organic solvent extraction of the anti-fungal compounds—The culture filtrate was extracted with different organic solvents and bioassayed against all the test fungi by agar well diffusion assay along with respective solvents as negative control. Among all, ethyl acetate extract was the most effective against various test fungi (Table 3), hence selected for chromatographic analysis. The preparative TLC plate developed in solvent system Di-chloro methane: Acetone (3:1) resolved a spot at Rf value 0.6, gave good anti-fungal activity against test pathogens. The HPTLC emission spectrum of the bioactive compound was recorded at 366 nm wavelength (Fig. 3). MS analysis revealed the estimated molecular weight of 315 Da (according to the M+1 rule) for the compound (Fig. 4).

Assessment of chitinase and β-1, 4-glucanase activity—The maximum chitinase activity exhibited by NII 1006 was recorded 0.05 Uml^{-1} on the fourth day of incubation as shown in Fig. 5 whereas the culture was found to be devoid of β-1,4-glucanase enzyme.

Effect of crude extracellular metabolite on the fungal and yeast morphology—SEM observations revealed that the test fungi *Botrytis cinerea* hyphae was deteriorated and broken into debris when treated with cell free crude supernatant of NII 1006 whereas the untreated hyphae exhibited a smooth surface indicating normal growth. Furthermore, similar results were obtained in case of yeast *Candida albicans,* where the untreated samples showed chain formation with smooth surface exhibiting normal growth, while the treated samples showed inhibition of chain formation as well as distorted morphology (Fig. 6).

Table 2—Cultural and morphological observations of *Streptomyces* sp. NII 1006 on various media

Media	Substrate mycelia	Arial mycelia	Spore formation
ISP1	Beige brown	Yellow tint	++
ISP2	Deep yellowish brown	Very light brownish green	+++
ISP3	Moderate olive brown	Very pale blue	++
ISP4	Dark yellow	Light yellow green	++
ISP5	-	-	-
ISP6	Dark brown	Very pale blue	++
ISP7	Moderate greenish yellow	Moderate greenish yellow	+
SC agar	Light yellow green	Light yellow green	-
TS agar	Dark yellow	Moderate greenish yellow	-

*Based on ISCC-NBS COLOR CHARTS standard sample No.2106 (Kelly 1964)

Effect of enzymatic treatment and temperature on activity of NII 1006 culture supernatant—The anti-fungal activity of cell free crude supernatant of NII 1006 remained unaffected when treated with lipase enzyme indicating no lipid moiety in the bioactive compound. There was a significant loss ($P \leq 0.05$) of anti-fungal activity of proteinase K treated crude supernatant as compared to untreated control against all the three test fungi used for bioassay (Fig. 7). Anti-fungal metabolites present in cell free crude supernatant of NII 1006 were heat stable when subjected to a range of temperature for one hour upto 70 °C. Further increase in temperature resulted in significant loss ($P \leq 0.05$) of bioactivity against all the test fungi used for bioassay (Fig. 8).

Effect of pH, incubation period, carbon and organic nitrogen source on culture NII 1006

The initial *p*H value ranging from 5.0 to 9.0 had no adverse effect on the stability of active metabolites while anti-fungal activity was absent at *p*H 4. The high biomass and anti-fungal metabolite production was found maximum when initial *p*H of the medium set at 7.0 as shown in Fig. 9. The anti-fungal activity as well as biomass was found to be highest at incubation period of 144 hrs (Fig. 10). Glucose used as carbon source resulted in high biomass yield, however, the bioactivity was maximum when arabinose was used as carbon source (Fig. 11 A). Among the tested nitrogen sources, peptone supported the highest biomass as well as anti-fungal metabolite production (Fig. 11 B).

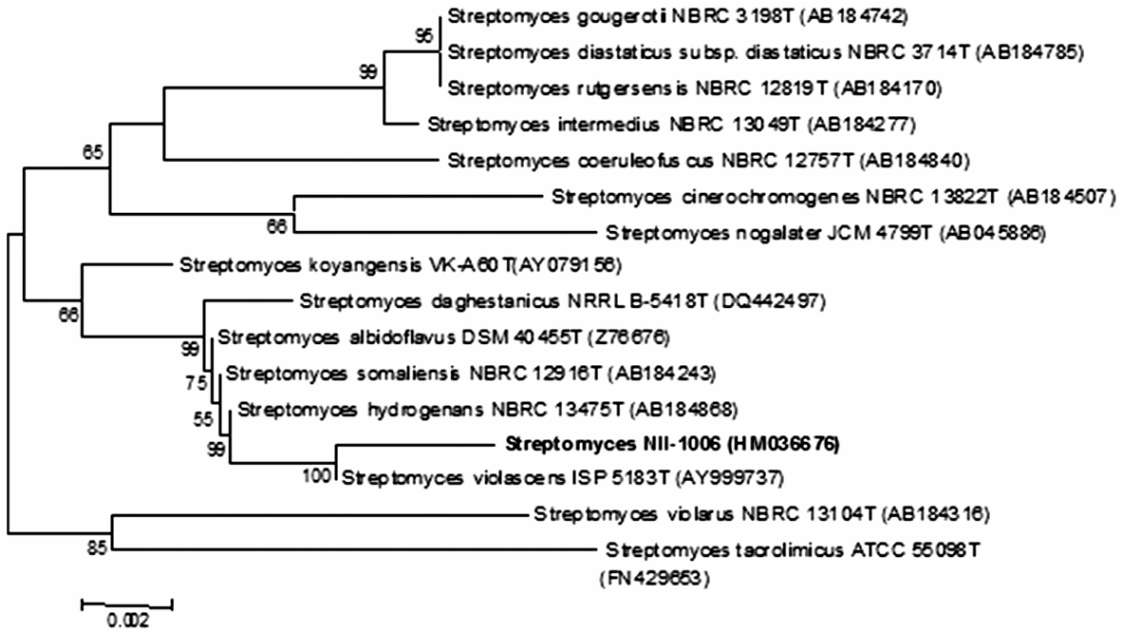

Fig. 2—Phylogenetic analysis based on 16S rRNA gene sequencing showing position of *Streptomyces* sp. NII 1006 in Neighbor-joining tree

Table 3—Inhibition of test fungi with different solvent extracts of NII 1006 ZOI (mm) [a]

Test fungi	Hexane extract	Ethyl acetate extract	Chloroform extract	Aqueous extract
Penicillium expansum	11±0.5	11±0.5	36±0.5	20±0.5
Aspergillus flavus	-	-	-	-
Rhizopus oryzae	11±0.5	11±0.5	15±0.5	-
Penicillium chrysogenum	-	14±0.5	14±0.5	-
Geotrichum candidum	17±0.5	22±0.5	15±0.5	-
Oidium sp.	22±0.5	31±0.5	17±0.5	-
Candida albicans	-	21±0.5	17±0.5	11±0.5
Aspergillus niger	11±0.5	12±0.5	-	-
Fusarium oxysporum	-	13±0.5	24±0.5	15±0.5
Botrytis cinerea	12±0.5	12±0.5	13±0.5	-
Phytophthora capsici	11±0.5	13±0.5	11±0.5	-

[a] Standard deviation (SD) calculated (n=3)

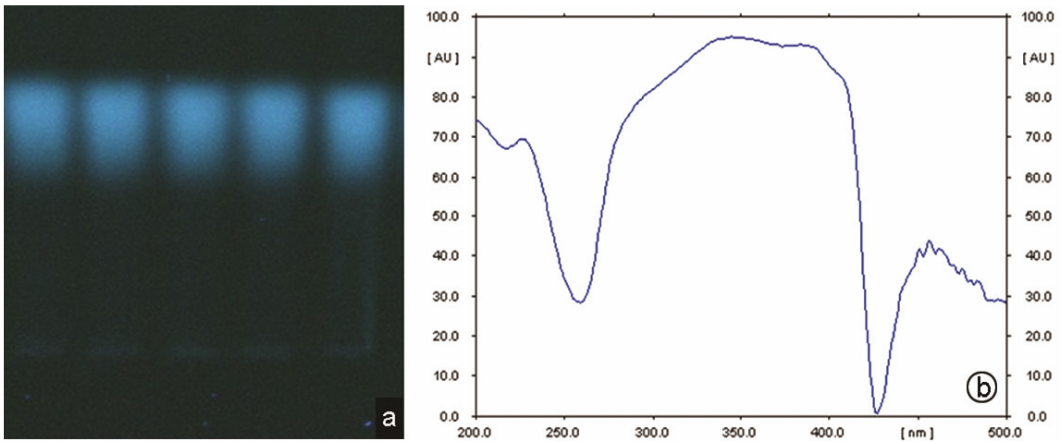

Fig. 3—(a) HPTLC profile of active spot with R_f value 0.6 developed in solvent system: Di-chloro methane: Acetone (3:1) at 366 nm. (b) HPTLC emission spectrum of active spot at 366nm wavelength

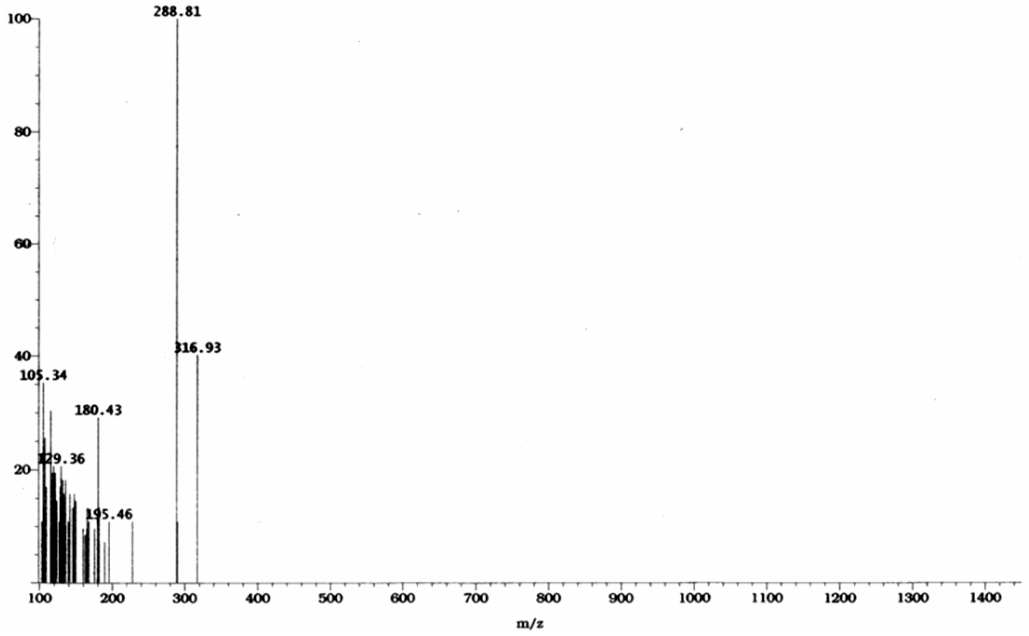

Fig. 4—Mass spectrometry analysis of bioactive compound

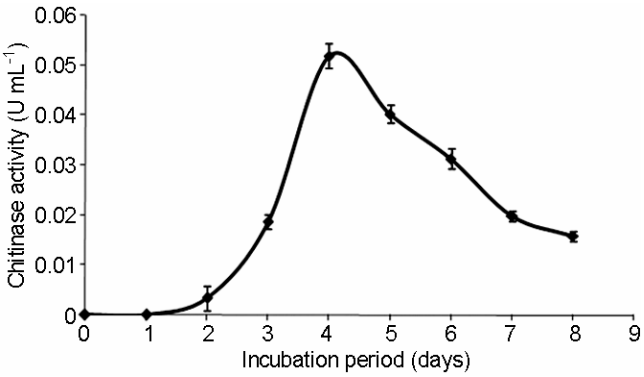

Fig. 5—Assessment of chitinase activity (U mL^{-1}) produced by NII 1006 with respect to incubation time

Discussion

Heavy loss of economically important crops by phytopathogens and its suppression by the exhaustive use of chemical fungicides are becoming key alarming situations in the Agri-sector. These problems may be sorted out by means of biocontrol where one species is capable of maintaining the population of the other species at low level. Actinomycetes have been widely explored for providing important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds [24]. Many species of actinomycetes are well established as anti-fungal biocontrol agents that inhibit several plant

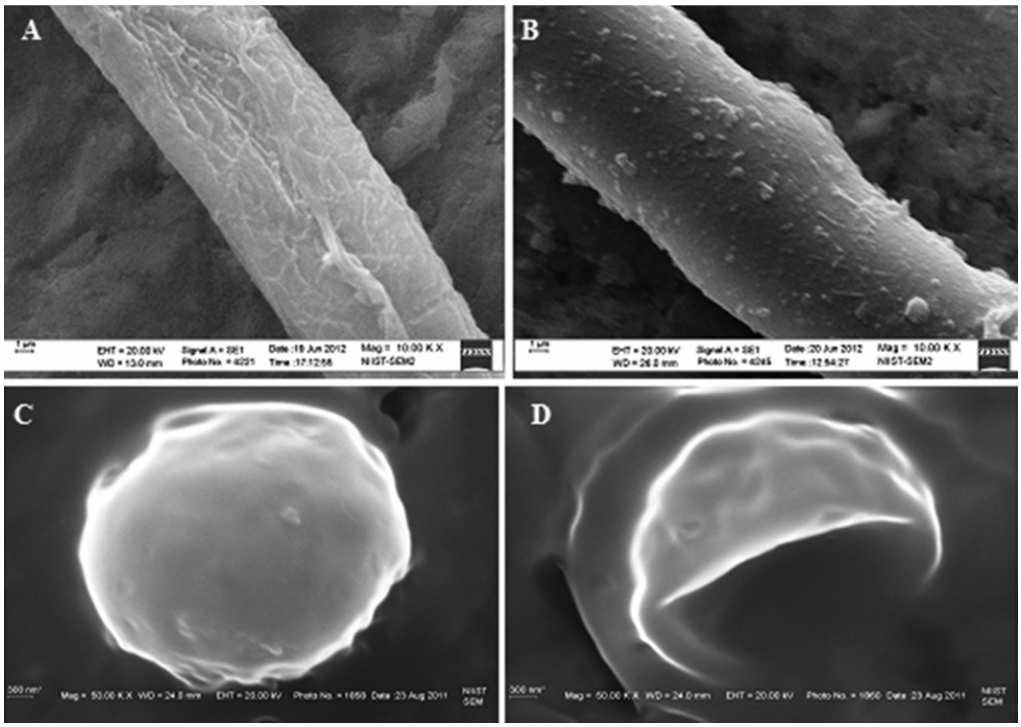

Fig. 6—Effect of crude extracellular metabolite on the test fungal and yeast morphology. (A) Morphology of untreated culture of *B. cinerea* showed the normal healthy hyphae, (B) Distorted morphology along with debris of fungal hyphae when treated with NII 1006 cell free supernatant rendered death of fungi indicated the inhibition, (C) Morphology of untreated culture of *C. albicans* indicated the normal cell shape, (D) *C. albicans* treated with NII 1006 cell free supernatant indicated deformation of cell shape and rendered the cell death

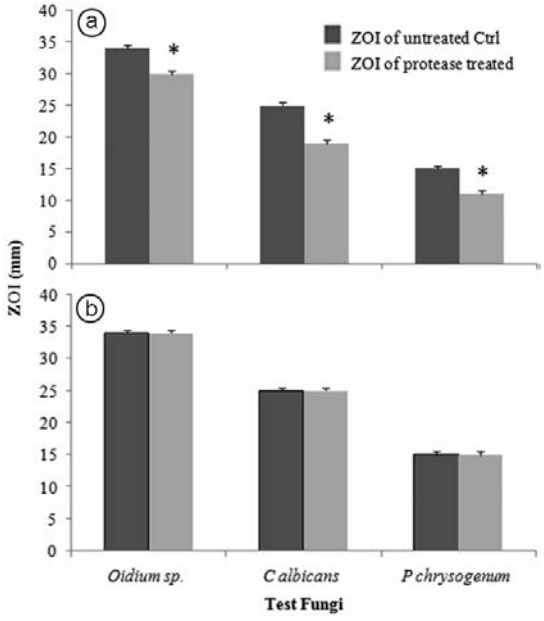

Fig. 7—(a) Effect of enzymatic treatment on bioactivity of culture supernatant NII 1006. Effect of proteinase K treatment on bioactivity of NII 1006 culture supernatant checked against three different test fungi indicated significant decline in its activity. (b) Effect of lipase treatment on the activity of NII 1006 culture supernatant checked against three different test fungi indicated, the activity remained unaffected

pathogenic fungi [2-4]. Various species of *Streptomyces* have previously been reported for biological control activity by means of producing various bioactive compounds as well as a variety of extracellular lytic enzymes [8,11,25-26].

The aim of the present study was isolation and screening of potent actinomycetes with biocontrol properties from the soil samples obtained from Silent Valley National park located in the range of Western Ghats of Kerala, known for its hotspot of biodiversity. Strain NII 1006 was one of the potential strains isolated, exhibiting anti-fungal activity against a wide range of test fungi. This study provides an assessment of *in vitro* anti-fungal activity of *Streptomyces* isolate NII 1006 attributed to the presence of chitinolytic enzyme and anti-fungal secondary metabolites. Further, the characteristics of cell free supernatant were evaluated by enzymatic treatment, heat treatment etc. Treatment with Proteinase-K showed a significant decrease in anti-fungal activity indicating the presence of lytic enzymes, in addition to anti-fungal secondary metabolites contributing bioactivity. The cell free supernatant when subjected to a range of temperature, also supported the same

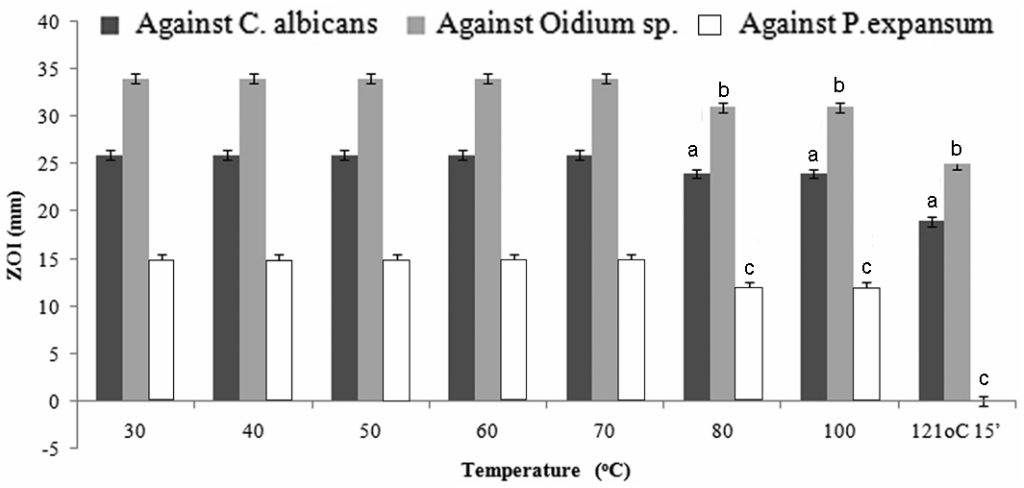

Fig. 8—Effect of temperature variation on stability of bioactive metabolite(s) present in culture supernatant of NII 1006. Culture supernatant was subjected to different temperatures and the bioactivity was analyzed against three different test fungi

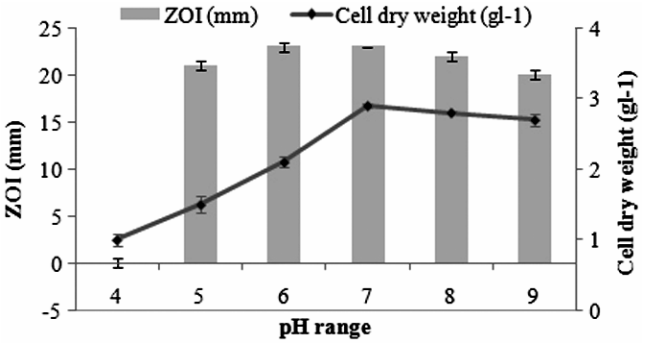

Fig. 9—Effect of *p*H variation on bioactivity and biomass production of NII 1006. The culture was grown at different *p*H (4-9) and analyzed for its bioactivity

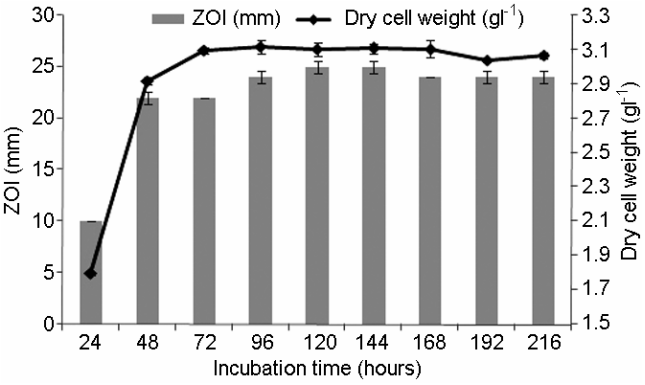

Fig. 10—Effect of incubation time on bioactivity and biomass production of NII 1006. The culture was incubated for different time intervals and the bioactivity was analyzed

indicating presence of heat labile lytic enzymes as well as heat stable anti-fungal compound(s) in the culture supernatant. Further, Chitinase activity was evaluated and found to be maximum at fourth day of incubation. This study is in agreement with the

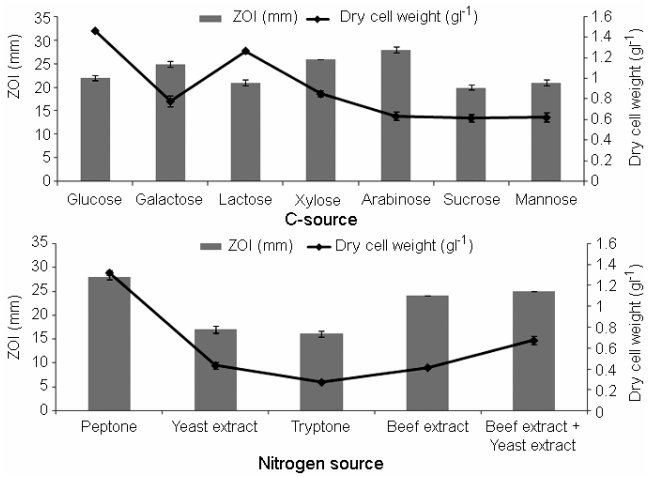

Fig. 11—Effect of various Carbon sources on bioactivity and biomass production of NII 1006. The culture was grown on basal medium with different Carbon sources and the bioactivity was analyzed. Effect of Nitrogen source on anti-fungal activity and biomass production of NII 1006. The culture was grown on basal medium with different Nitrogen sources and the bioactivity was analyzed

findings of Prapagdeé *et al.*[27] wherein, the anti-fungal activity in exponential phase was mainly due to extracellular chitinase enzyme whereas in the stationary culture filtrate the bioactivity involved the action of unknown thermostable anti-fungal compound(s). Our results are also in consensus with the investigations of Lee *et al.*[28] in which biocontrol mechanism of *S. cavourensis* SY224 was associated with the production of Chitinase, *β*-1,3 Glucanase, and 2-Furancarboxaldehyde.

The attempts were done to purify the bioactive compound as the compound characterization might explore the ideas for its effective application strategies in *in vivo* experiments. The anti-fungal compound was

partially purified by HPTLC, however, further purification and chemical characterization of bioactive metabolite(s) is in progress. An evaluation of the disease control efficacy of the strain NII 1006 would further be conducted under green house and field conditions.

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