Indian Journal of Biotechnology
Vol. 17, April 2018, pp 261-271

Optimization and characterization of antifungal metabolite from a soil actinomycete *Streptomyces indiaensis* SRT1

Bhosale H J[1]*, Kadam T A[1], Mirajgave R S[1] and Holkar S K[2]

[1]School of Life Sciences, Swami Ramanand Teerth Marathwada University, Nanded 431606, India

[2]Hislop School of Biotechnology, Hislop College, Nagpur 440001, India

Received 14 July 2014; revised 19 May 2016; accepted 18 October 2016

A total of 77 soil actinomycete isolates were screened for antifungal activity by cross streak method and agar well diffusion assay. The potential producer strain AI-32 was identified as *Streptomyces indiaensis* SRT-1 based on its morphological, cultural and physiological characteristics and 16S rRNA gene sequencing. The antifungal metabolite from AI-32 was extracted with organic solvents and purified by column chromatography. Its structure was elucidated by UV, FTIR, NMR and LC-MS analysis and was found to contain an aromatic ring fused with a furan ring linked by ester linkages. The culture conditions for the production of antifungal metabolite were optimized by using various physical and chemical parameters. The optimization studies revealed soybean casein digest supplemented with 1% dextrose (SCDD) broth as the suitable production medium, pH as 8.5, temperature (30^{o}C), salinity (2%), and incubation period as 8 days and glucose and soybean meal as suitable carbon and nitrogen sources, respectively. Based on the minimum inhibitory concentration and minimum fungicidal concentration values, *Fusarium oxysporum* (NCIM 1072) was found to be more sensitive test pathogen. The effect of antibiotic on spore germination and mycelial development of *F. oxysporum* was determined in terms of delayed spore germination, appearance of segmented mycelium and ruptured and distorted spores. This study is the first report highlighting the significance of *S. indiaensis* produced metabolite as a promising antifungal agent.

Keywords: *Streptomyces indiaensis*, antifungal, spore germination, mycelial development, *Fusarium oxysporum*

Introduction

Fungal phytopathogens pose serious complications worldwide in the cultivation of economically important crops due to the plant diseases caused by them. Plant diseases caused by fungi include rusts, smuts, rots, and may cause severe damage to crops. Chemical fungicides are extensively used in current agriculture to control fungal pathogens. However, the extensive use of chemical fungicides in agriculture is associated with deleterious effects on human health, environmental pollution and development of pathogen resistance to fungicides. Thus, many researchers have focused on biological control of plant diseases through antagonistic microorganisms, and this management of plant disease is greatly considered as an alternative to use of chemical fungicides[1]. Generally, mechanisms through which antagonistic microorganisms inhibit fungal pathogens are antibiosis, iron competition, parasitism, and/or a synergistic combination of these modes of action[2].

Actinomycetes are the most ecologically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive metabolites including antibiotics[3], antitumor and immunosuppressive agents[4] and enzymes[5]. Among actinomycetes, *Streptomyces* species are known as most promising candidates as they are producers of about more than 80% of secondary metabolites identified from them. The biocontrol potential of many Streptomycetes has often been linked to their ability to produce antibiotics[6]. However, the immense biotechnological potential of actinomycetes has led to extensive search on isolates from normal terrestrial habitats and resulted in an associated increase in the number of compounds being rediscovered due to a high rate of redundancy in the strains isolated[7]. A mathematical model designed to estimate the number of undiscovered antimicrobial agents from the genus *Streptomyces* predicts that the decline of finding such agents is due to the declining efforts in screening more microorganisms and not due to a lesser number of undiscovered antimicrobial agents[8] and thus, the search for microorganisms and new

*Author for correspondence:
Tel: 02462-229300; Fax: 02462-229242
bhoslehemlata@gmail.com

antibiotics has gained importance. Hence, to expect the discovery of new bioactive compounds, it becomes important to search for actinomycetes from less explored or untapped ecosystems. The present work describes the isolation, production optimization, purification, preliminary structural elucidation of an antifungal agent produced by a newly isolated *Streptomyces indiaensis* from soil of Marathwada regions, which are less exploited in drug screening programs. Further, the effect of metabolite on spore germination and mycelium development in *F. oxysporum* was determined.

Materials & Methods

Soil Sampling

A total of 53 soil samples were collected from agricultural fields of Nanded (19°09'N, 77° 27'E), Latur (18.4°24'N, 76°36'E), Parbhani (19°08'N, 76°5'E) and Parli (19°08'N, 76°5'E) regions of Marathwada (M.S), India and used for the isolation of actinomycetes. The soil samples (20 g) were air dried for 72 h and 1 gm of each soil sample was suspended in 100 mL of sterile distilled water and homogenized by vortexing. The treated samples were serially diluted up to 10^{-6} and spread (0.1 mL) over the surface of starch casein agar (SCA) medium[9] supplemented each with 50 µg/mL cycloheximide, nystatin and nalidixic acid. The plates were incubated at 30°C for 7-10 days. Seventy seven morphologically distinct isolates were obtained during the course of our investigation. All these isolates were screened for their antifungal activity against phytopathogenic fungi.

Test Organisms

Sclerotium rolfssi (NCIM 1084), Ustilago maydis (NCIM 983), *Rhizopus oryzae* (NCIM 878), *Mucor hiemalis* (NCIM 873), *Helminthosporium graminum* (NCIM 1070), *Fusarium oxysporum* (NCIM 1072), *Aspergillus fumigatus* (MTCC 2544), *Aspergillus flavus* (NCIM 1028), *Aspergillus niger* (NCIM 586), *Fusarium moniliformis* and *Alternaria solani* isolated from infected tomato plants were used to study antifungal activity of actinomycetes isolates.

Screening for Antifungal Activity

Antifungal activity of isolates was tested primarily by cross streak method[10]. The isolates showing positive activity were used for secondary screening of antifungal activity. The actinomycetes isolates were inoculated in 50 mL of starch casein (SC) broth and incubated for 7 days at 30°C . After incubation, the culture broths were centrifuged at 15000 × g for 20 min and cell free supernatants (CSF, 0.1 mL) were used to observe antifungal activity by agar well diffusion assay[11]. The antifungal efficacy of the isolates was estimated on the basis of the zones of inhibition obtained. Each experiment was performed in triplicate and the results were expressed as an average. The isolate AI-32 showing strong antifungal activity was selected for further study.

Characterization of AI-32 Isolate

Morphology and pattern of sporulation of isolate AI-32 was observed under light microscope using inclined cover slip culture techniques[12] on SCA after incubating at 30°C for 7 days. The spore surface ornamentation was examined by scanning electron microscope. Color of spore mass and aerial and substrate mycelia were recorded using National Bureau Standards (NBS) color name charts[13]. Cultural characterization was done by growing the isolate on yeast extract malt extract agar, starch casein agar, oatmeal agar, glycerol asparagine agar, peptone yeast extract iron agar, Bennet's agar and tyrosine agar at 30°C for 7 days. All media and media ingredients were purchased from HiMedia, Mumbai (India). The growth of organism was studied at different temperatures viz, 25°C, 30°C, 35°C, 40°C and 45°C and pH 5.0, 6.0, 7.0, 8.0 and 9.5. Utilization of different carbon sources (D-glucose, L-arabinose, D-xylose, D-mannose, D-fructose, D-galactose, mannitol, sucrose) and nitrogen sources (L-cysteine, L-histidine, L-leucine, L-phenylalanine and L-valine) was studied using starch casein broth as basal medium. Chemotaxonomic studies were done by analyzing the whole cell hydrolysates for amino acids and sugar content[14,15].

The identity of the AI-32 isolate was confirmed by 16 S rRNA sequencing. The resultant gene sequences were aligned with the sequences available at GenBank database by using BLAST software. The sequence obtained was submitted to NCBI GenBank database and sequence accession number was obtained.

Production and Purification of Antifungal Compound

Soybean casein digest broth supplemented with 1% dextrose (SCDD) was used for optimum production of antifungal antibiotic from AI-32. 2 L sterilized medium was inoculated with heavy spore suspension of AI-32 and incubated in a rotary shaker at 200 rpm for 8 days at 30°C. At the end of incubation period, the fermented

broth was centrifuged at 10,000 × g for 30 min to separate biomass and used for the extraction of antifungal metabolite.

The effect of different solvents on extraction of antibiotic from supernatant was studied. Six different solvents such as ethyl acetate, chloroform, methanol, n-butanol, hexane and diethyl ether were used on the basis of polarity. All extracts were assayed for their antifungal activity using agar well diffusion assay using respective controls. The active solvent extract was evaporated in a rota vapor (Superfit) at 40°C under vacuum. The dark brown sticky residue was obtained, resuspended in methanol and concentrated to obtain crude antibiotic powder. The methanolic solution of powder (1.5 g) was chromatographed on silica gel (60-120 mesh) column using gradient of ethyl acetate in water (0-100%) as eluent. Fifty fractions were collected (1 ml min^{-1}) and each fraction was tested for its activity using agar well diffusion assay. The active fractions were combined and evaporated to dryness in vacuum to give 140 mg of the compound.

The purified compound was analyzed for the number of components by thin layer chromatography (TLC) (ethyl acetate : acetone : water; 2 : 3 : 1). After development, the spots were located with iodine vapors. The antifungal activity of the active components on TLC plates was further confirmed as mentioned earlier. The fractions showing similar Rf values were pooled together, purified by column chromatography (as mentioned above) and used for further characterization.

Structural Characterization

Ultraviolet (UV) spectrum was recorded on spectrophotometer (Shimadzu, UV-VIS 2100 Spectrophotometer). One hundred microgram of sample was dissolved in 5 ml of ethanol and the spectra were recorded within 200 – 800 nm range. The infrared spectra were recorded on FTIR Spectrophotometer (Shimadzu, FTIR Affinity 1, Japan). The spectra were obtained using KBr pellet method in the 400 - 4000 cm^{-1} scanning range. The melting point was determined using method adopted by Dhanasekaran *et al*[11]. For nuclear magnetic resonance (NMR) studies, 0.3 mg of antibiotic sample was dissolved in 3 ml of dimethyl sulfoxide and analyzed on a Varian 400 MHz NMR spectrometer. The liquid chromatography mass spectrum (LCMS) was obtained from HP CHEM mass spectrometer.

Optimization of Growth and Antifungal Metabolite Production

Effect of Media

Seven different broth media namely, yeast extract malt extract (YEME) medium, SC broth, oatmeal medium (OMM), glycerol asparagine medium (GAM), peptone yeast extract iron medium (PYEIM), Bennet's medium and SCDD were inoculated with AI-32 culture and incubated at 30°C for seven days under shaking conditions. After incubation, the biomass was separated by centrifugation at 10,000 rpm for 20 min and dried at 70°C until a constant weight was obtained and expressed as mg/100 ml[16]. The ethyl acetate extract of antibiotic was prepared and tested for antifungal activity against *F. oxysporum* as mentioned previously.

Effect of Carbon and Nitrogen Sources

The soybean casein digest broth supplemented with 17 different carbon sources (10 g/l) such as glucose, fructose, maltose, starch, xylose, sucrose, mannitol, inositol, arabinose, rhamnose, cellobiose, lactose, salicin, galactose, defatted groundnut powder (DGP), defatted soybean powder (DSP) and cellulose and 8 nitrogen sources (10 g/l) including yeast extract, malt extract, soybean meal (SBM), KNO_3, peptone, ammonium sulphate, asparagine and casein were inoculated separately with active culture of AI-32. The flasks were incubated for 7 days under shaking conditions and after incubation, the biomass and antifungal effect were evaluated as mentioned earlier.

Effect of Incubation Periods

The SCDD broth was inoculated with AI-32 and incubated for 12 days. At every 24 h interval, the effect on growth and antifungal activities were tested as stated earlier.

Effect of Temperature

The AI-32 was inoculated into SCDD broth and incubated at different temperatures viz. 20, 30, 40, 50 and 60°C for seven days. After incubation, the broth was analyzed for biomass and antifungal activity.

Effect of pH

The pH of the SCDD broth was adjusted to 4, 5, 6, 7, 8, 9 and 10 with 0.1 M NaOH or HCl. All the flaks were inoculated with AI-32 and incubated at 30°C for seven days. The biomass and antifungal effect was determined as stated earlier.

Effect of NaCl

The SCDD broth was supplemented with different NaCl concentrations (1-20%) and inoculated with AI-32 culture. The flasks were incubated at 30°C for seven days under shaking conditions and subjected for biomass and antifungal activity determination.

Determination of MIC and MFC

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of the antibiotic were determined against all test pathogens by micro dilution method in culture broth as previously described[4]. The antifungal compound was dissolved in ethyl acetate to obtain a concentration of 100 µg/ml. Different concentrations (10-100 µg/ml) were used to evaluate the efficacy of antifungal metabolite against all test pathogens.

In vitro* Antagonistic Activity of AI-32 Metabolites on Mycelia Development and Spore Germination of *F. oxysporum

The test fungus was grown on potato dextrose agar (PDA) plates at 28°C for 48-72 h. Fungal mycelium was aseptically placed on the surface of microscopic slides covered with PDA containing 10 µg/ml of antifungal compound. The inoculated slides were incubated at 28°C in the dark for 72 h. The fungal hyphae were stained with lactophenol cotton blue staining solution, and subsequent changes in the morphology of fungal hyphae were observed using a light microscope (Olympus, 40X). To study the effect of metabolite on spore germination, 100 µl fungal spore suspension (prepared in distilled water) was added to 100 µl of antibiotic (20 µg/ml in ethyl acetate), in glass vials and incubated at 28 ± 2°C for 24 h. The control vials contained ethyl acetate in place of antibiotic. After incubation, the content of the vials was stained with cotton blue and mounted in lactophenol. The spores were observed under a microscope for their germination status and inhibition of spore germination (%) was calculated[17].

Results and Discussion

In the present study, a total of 77 morphologically distinct actinomycetes were isolated from soil and screened for antifungal activity. It was found that 45 (58.4%) isolates showed inhibition of one or more test fungi (data not shown). The most promising isolate AI-32 showing broad and comparably more antifungal activity than other actinomycetes was selected for further study (Table 1, Fig. 1). All test pathogens except *Sclerotium rolfssi* (NCIM 1084) were sensitive to AI-32. The isolate showed highest zone of inhibition (36 mm) against *Fusarium oxysporum* (NCIM 1072) and least activity (15 mm) was observed against *Mucor hiemalis* (NCIM 873) whereas other test pathogens showed moderate sensitivity to AI-32. The isolate AI-32 was found to belong to genus *Streptomyces* on the basis of its morphology, cell wall analysis and cultural, physiological and biochemical features. The isolate grew well on most media except on oatmeal agar. The color of spore mass varied from white, pink, grey to dark brown depending on the medium used (Table 2). The isolate was Gram positive and produced light pink colonies on Bennet's agar with dark brown colony reverse (Fig. 2). It showed highly branched substrate mycelia, aerial hyphae and long spore chain under light microscope. The presence of rectiflexible spore chain was revealed by scanning electron microscopy. The whole cell hydrolysate of AI-32 showed the presence of LL- diaminopimelic acid and was without any characteristic sugar indicating a type I cell wall chemotype (Table 3). The morphological and chemo typing studies of AI-32 revealed its position in genus *Streptomyces*[12]. Its further

Table 1 — Antifungal activity of AI-32 in the secondary screening

S. No	Test organism	Zone of inhibition (mm)
1	*Rhizopus oryzae* (NCIM 878)	17 ± 0.5
2	*Ustilago maydis* (NCIM 983)	19 ± 1.2
3	*Sclerotium rolfssi* (NCIM 1084)	ND[a]
4	*Mucor hiemalis* (NCIM 873)	15 ± 0.9
5	*Fusarium moniliformis*	16 ± 0.5
6	*Fusarium oxysporum* (NCIM 1072)	36 ± 1.0
7	*Aspergillus fumigatus* (MTCC 2544),	25 ± 0.5
8	*Aspergillus flavus* (NCIM 1028)	23 ± 0.7
9	*Aspergillus niger* (NCIM 586)	23 ± 1.4
10	*Alternaria solani*	17 ± 0.4
11	*Helminthosporium graminum* (NCIM 1070)	22 ± 0.9

[a]Not detected

Each value is an average of three replicates and represented as mean ± S.D.

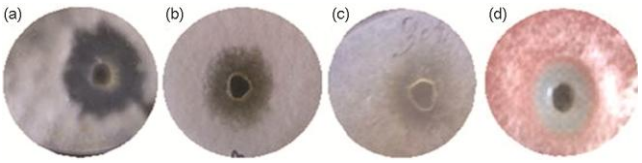

Fig. 1 — Antifungal activity exhibited by crude extract of *Streptomyces* AI-32 against a) *Ustilago maydis* b) *Alternaria solani* c) *Rhizopus oryzae* and d) *Fusarium oxysporum*.

Table 2 — Cultural characteristics of AI-32 Isolate

Growth medium	Growth[d]	Spore mass color	Colony reverse
Bennets agar	+++	Pink	Brown
Starch casein agar	+++	White	Pale yellow
YEME agar[a]	+++	White	Brown
Oatmeal agar	+	Grey	Cream
GA agar[b]	++	White	-
PYE iron agar[c]	++	Dark brown	Black brown
Tyrosine agar	++	Grey	-

[a] Yeast extract malt extract agar, [b] Glycerol aspargine agar, [c] Peptone yeast extract iron agar, [d]+++ excellent growth, ++ good growth + scarce growth

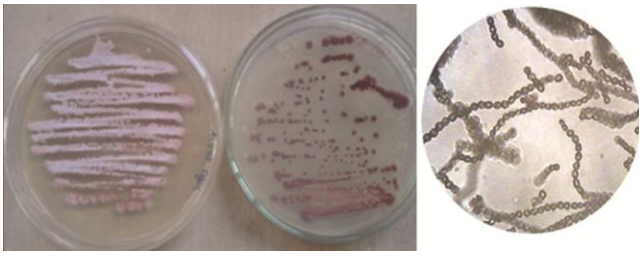

Fig. 2 — *Streptomyces* AI-32 on Bennets agar a: Light pink colonies with mycelium, b: Deep pink to brown colony reverse and micrograph showing spore chain of AI-32 (c).

characterization was done on the basis of its 16S rRNA sequencing. The similarity level of the isolate was 99% with *Streptomyces indiaensis* NBRC 13964 and hence, was identified as *Streptomyces indiaensis* SRT-1. The 16S rRNA sequence of the strain AI-32 as *Streptomyces indiaensis* SRT-1 (1171 nucleotides) has been deposited in GenBank under the accession number JQ065726. The streptomycetes are the producers of more than half of the 10,000 reported bioactive compounds and have offered increasing interest to industry and academics since last 50 years[18]. However, the antifungal property of *S. indiaensis* has not been revealed and reported earlier. We strongly suggest this as the possibly the first report on antifungal activity of *S. indiaensis.*

In the present study, *S. indiaensis* SRT-1 could produce detectable quantities of antifungal compounds in soybean casein digest broth (SCDB) supplemented with 1% dextrose. The fermented broth of *S. indiaensis* was extracted with five different solvents. The maximum antibiotic yield was obtained using ethyl acetate as extractant (18.89 mg/L) followed by chloroform and diethyl ether (Table 4) whereas it could not be dissolved in methanol, n-butanol and n-hexane. This is in accordance with earlier reports where most of the antifungal antibiotics are extracted with ethyl acetate[19,20]. The purification of ethyl acetate extract of antibiotic on silica gel column revealed that the fractions 15-23 are the major

Table 3 — Morphological and biochemical characteristics of AI-32 isolate

S. No.	Test character	Result
1	Morphological feature	Aerial mycelium with long chain of spores
2	Spore mass color	Light pink (#Ff7E93)
3	Colony reverse	Deep pink-brown (#F3545E)
4	Spore wall	Rectiflexible
4	Cell wall chemotype	I
5	Diagnostic amino acid in whole cell hydrolysate	LL-DAP
6	Diagnostic sugar in whole cell hydrolysate	NC
7	Temperature for growth (°C)	
	Range	20-50
	Optimum	30
8	pH for growth	
	Range	6.5-9.5
	Optimum	7.5
9	Nitrate reduction	Negative
10	Melanin formation	Negative
11	H_2S production	Positive
12	Degradation of	
	Casein	Positive
	Hippuric acid	Negative
	Esculin	Positive
	Hypoxanthine	Negative
	Tyrosine	Negative
	Xanthine	Positive
13	Utilization of carbon source	
	Glucose	Positive
	Arabinose	Negative
	Sucrose	Positive
	Xylose	Positive
	Mannose	Positive
	Mannitol	Positive
	Fructose	Positive
14	Utilization of nitrogen source	
	Valine	Negative
	Cysteine	Negative
	Phenyl alanine	Positive
	Histidine	Positive

bioactive fractions. The column purified active fraction showed single resolved band in thin layer chromatography and had Rf value of 0.83 cm.

The compound obtained from *S. indiaensis* was light brownish in color having melting point of 230°C. The UV spectrum of the compound showed absorption maxima at 270 nm in ethanol (Fig. 3). The FTIR spectrum exhibited broad absorption peak between 3100-3500 and a peak at 3032 cm^{-1}. The spectrum indicates that the compound had N-H stretch and aromatic C=CH group. The presence of C=O (lactone) stretch, C-H stretch and ether and ester groups confirmed by the bands present in the region of 1735 cm^{-1}, 2852.72 - 2922.16 cm^{-1} and 1462 - 1022 cm^{-1} respectively (Fig. 4). H1 NMR (500 MHz) spectrum of the antibiotic in dimethyl sulfoxide (DMSO) has peaks in the region of 1.2 to 10.4 δ. The aromatic protons peak appear as multiplet in between δ 7.0-8.68, furan proton at 6.2, singlet of $–NH_2$ proton at δ 3,9, triplet of $–CH_2$ appears at δ 1.9 and quartet of $–CH_3$ appear at δ 0.9 (Table 5). The LC-MS analysis showed a molecular ion peak at m/z 663.75 (Fig. 5). On the basis of the above spectral data, the compound was found to have an aromatic ring fused with a furan ring linked by ester linkages and identified as 2-amino 3-ethyl benzofuran (Fig. 5c). The isolation and characterization of furan containing secondary substances from S*treptomyces* sp. including antifungal compound 2, furan carboxyaldehyde by *S. cavourensis*

Table 4 — Extraction of antibiotic from *S. indiaensis* SRT-1

S. No	Solvents used for extraction of antibiotic	Yield of antibiotic (mg/l)
1	Ethyl acetate	18.89
2	Chloroform	10.22
3	Methanol	-
4	n- butanol	-
5	Diethyl ether	0.43
6	n-hexane	-

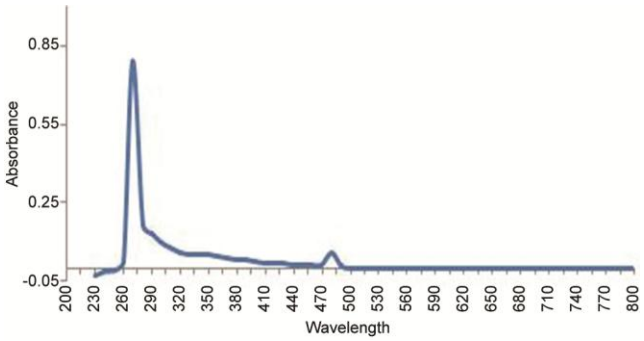

Fig. 3 — UV visible absorption spectrum of the purified fraction recorded in ethanol against ethanol blank. The fraction exhibited characteristic peak in the range of 270 nm.

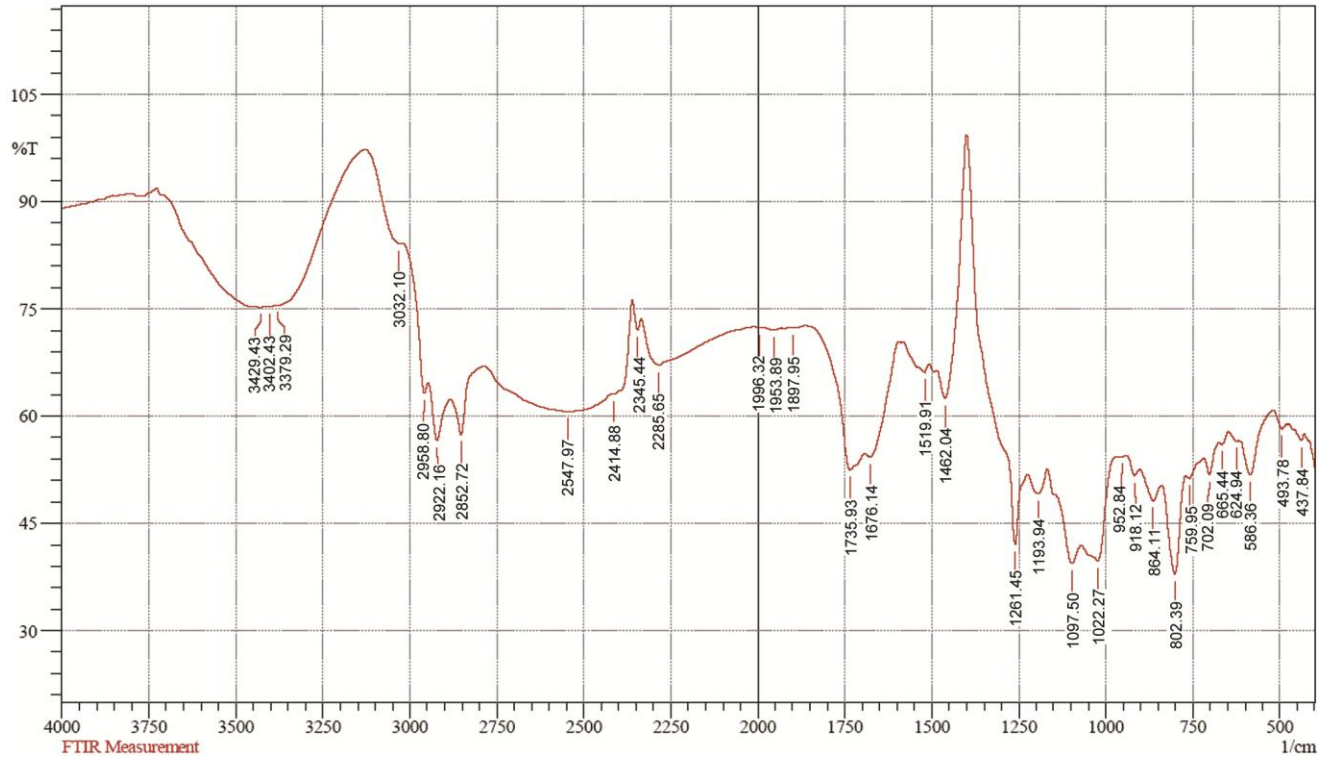

Fig. 4 — FTIR measurements of antifungal metabolite of *S. indiaensis* SRT-1.

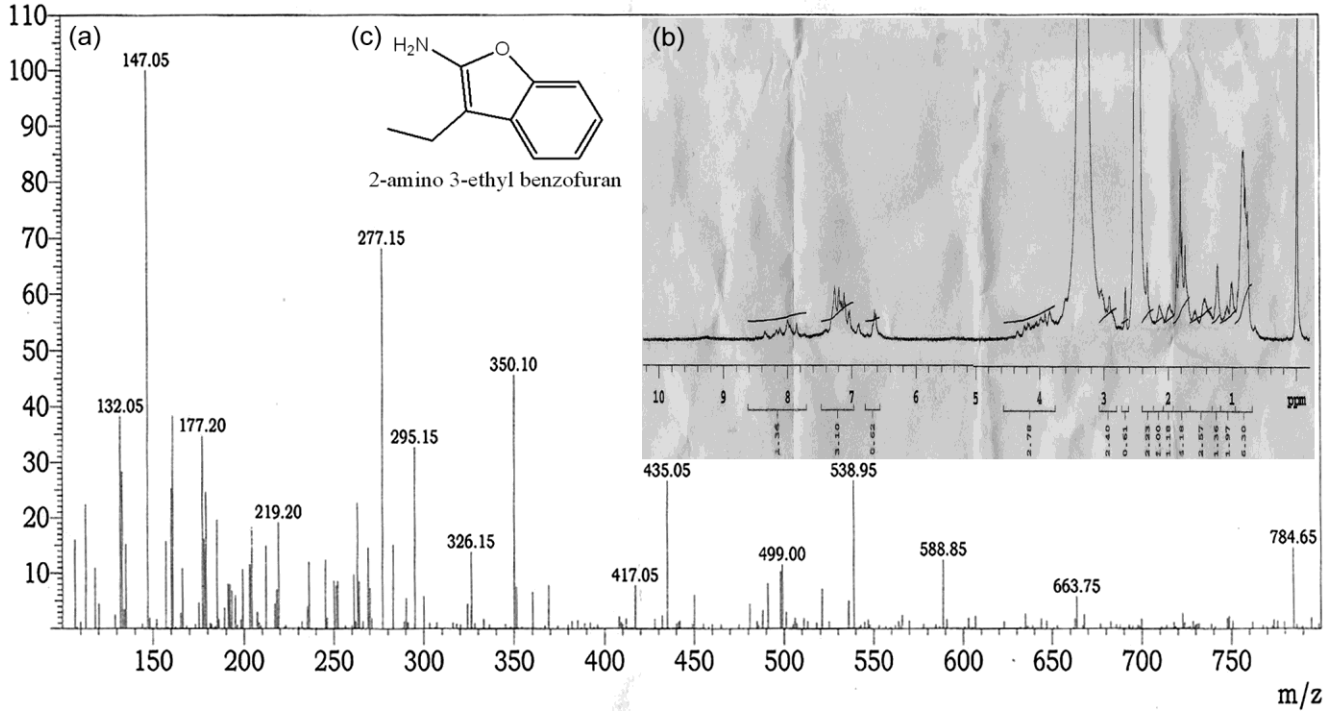

Fig. 5 — LC-MS (a), ^{1}H NMR (b) analysis and chemical structure of antifungal metabolite of *S. indiaensis* SRT-1.

Table 5 — Physico-chemical properties of the antifungal antibiotic from *S. indiaensis* SRT-1

S. No	Properties	Results
1	Color	Light brown
2	Melting point	230°C
3	Rf value	0.81
4	UV λ max (nm)	270
5	IR (KBr) cm-1	3379, 2922, 3032.10, 1670- 1740,
6	LC-MS (m/z)	1097-1022
7	H^1NMR	663.75
8	C-13 NMR	aromatic proton, furan proton, -NH_2, -CH_2 & –CH_3 proton
9	Elemental analysis	C-H, CH_2, CH_3 C-74.51, H-6.88, N-8.69, O-9.99

SY224[21], a dominant fungal growth promoting substance, azofuran by *Streptomyces* strain AcH 505[22] have been reported previously. Our studies are in contrast with these reported compounds with respect to the broad spectrum and strong antifungal activity.

It is absolutely necessary to have a complete knowledge on the optimal conditions required for the growth of producer strain and product formation for development of an efficient fermentation process for the production of secondary metabolites. In the present study, the conditions required for optimal growth and production of antifungal compound using *S. indiaensis* have been optimized using different physical and chemical parameters. Among the seven media tested, the strain showed maximum growth (329 mg/100 ml) and highest antifungal activity (21 mm) against *F. oxysporum* (Fig. 6a) on SCDD medium. Also, good growth and antifungal activity was observed in SCB, BB and YEME medium. Inoculation of strain in GAM and PYEIM showed moderate growth and antifungal compound production whereas lowest growth and inhibitory activity was observed when OMM was used. The variation in the antifungal metabolite production among different media could be due to variation in composition of the medium in which strain was grown. Vijayakumar *et al*[23] also reported that *Streptomyces* sp. VPTS3-1 grown on starch casein medium showed good antimicrobial activity against *Proteus vulgaris* and *Klebsiella pneumoniae* compared to asparagine-mannitol broth, ISP medium 2, ISP medium 4, ISP medium 5, KenKnight medium, nutrient medium and starch nitrate medium.

The type and amount of carbon and nitrogen sources play key roles in growth, deciding the nature of structural and energy compounds and act as direct precursors for antibiotic production in microorganisms. Hence, in the present study effect of various carbon and nitrogen sources on growth and antibiotic production was determined. All the carbon

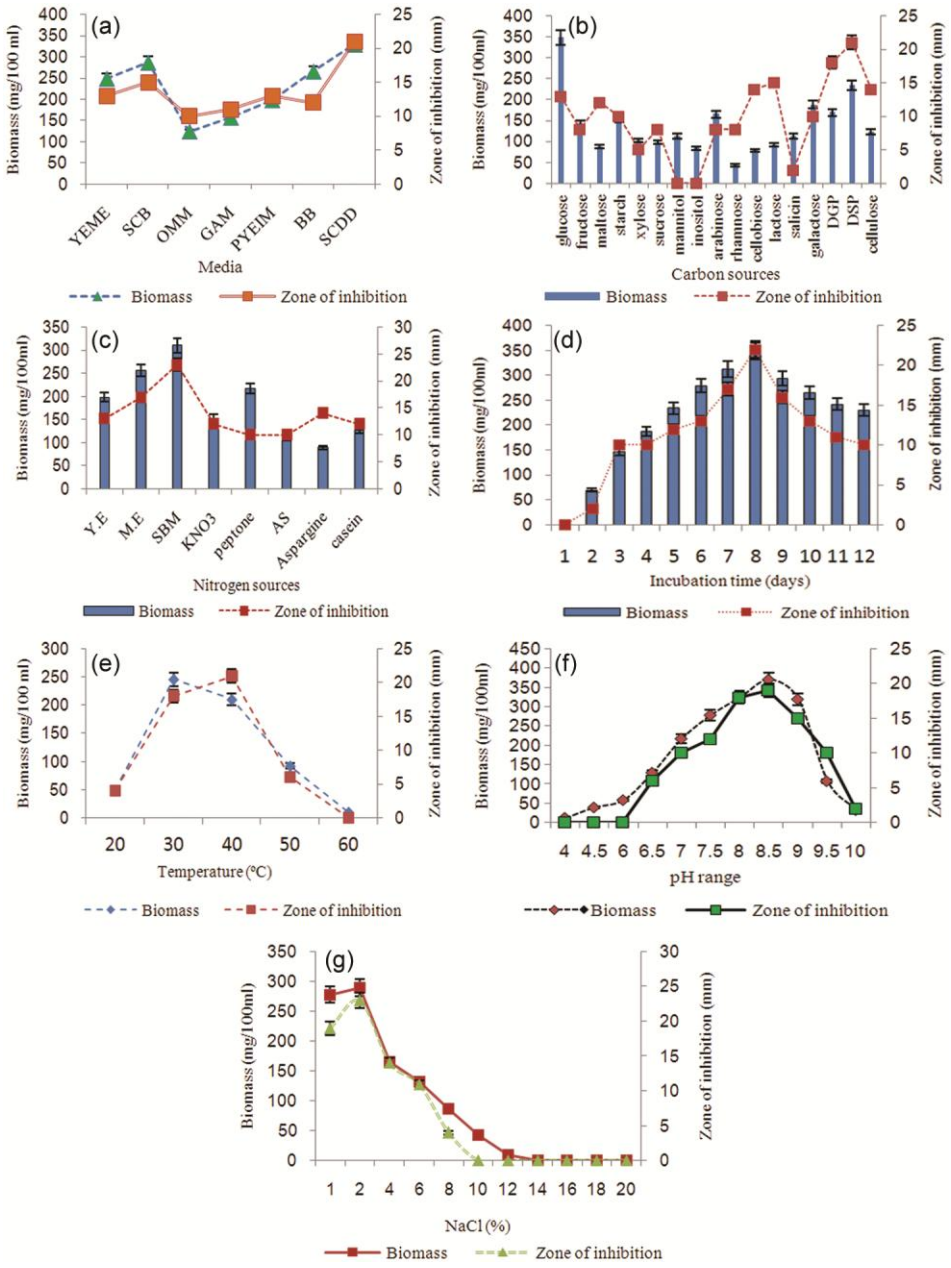

Fig. 6 — Effect of media (a) carbon sources (b) nitrogen sources (c) incubation time (d) temperature (e) initial pH of medium (f) varying NaCl concentrations and (g) on growth and antibiotic production by *S. indiaensis* SRT-1.

sources tested, supported the growth of the isolate. Glucose proved to be the best carbon source for cell growth (348 mg/100 ml) followed by DSP (234 mg/100 ml) and galactose (187 mg/100 ml) whereas, DSP was the most effective source for antibiotic production (21 mm) followed by DGP (18 mm) and lactose (15 mm). The isolate showed maximum cell growth in presence of glucose but it did not support antibiotic production (Fig. 6b). The possible explanation for this effect may be that glucose causes catabolite repression in which production of enzymes necessary for biosynthesis of secondary metabolites is inhibited[24]. Monosaccharides have been reported to be suitable substrates for growth but not for biosynthesis of antibiotics whereas production of secondary metabolite is often stimulated by slowly assimilated complex substances. Optimal production has been achieved by cultivating organisms in media containing slowly utilized nutrient sources such as glycerol[25] and starch[23].

Among the nitrogen sources tested, SBM supported maximum growth (310 mg) and antibiotic production (23 mm) in *S. indiaensis* SRT-1 followed by malt extract and yeast extract (Fig. 6c). This is in accordance with Vahidi *et al*[26] and Vijayakumar *et al*[23] who reported that organic nitrogen sources are superior for antibiotic production.

The growth and antifungal activity of *S. indiaensis* SRT-1 was observed from the second day of incubation and reached maximum after eighth day in SCDD medium. The progressive increase in biomass and size of inhibition zone was observed during the first 2-8 days of incubation and decreased gradually thereafter. It is evident from Figure 6d, the growth reached maximum on 8th day (349 mg) and the strain produced highest inhibition zone (22 mm) during this incubation period. The decrease in growth of the strain later can be related to the exhaustion of nutrients in media and accumulation of toxic by-products indicating unfavorable conditions for antifungal compound production. Kathiresan *et al*[27] optimized the required incubation time for the antifungal effect of the actinomycetes against *Fusarium solani* and found that it was suppressed with the increase in incubation period. The maximum inhibition was found with cultures incubated for 120 h. Similarly, Sejiny[28] reported that antibiotic production usually starts on 2 or 3 days but maximum activity is recorded on 9 or 10 days, that is, in stationary phase.

Temperature affects the physiology, morphology, biochemistry and metabolite production by microorganisms and each strain has an optimum temperature at which they grow maximally. In the present study, *S. indiaensis* SRT-1 produced maximum biomass (246 mg/100 ml) and had highest antifungal activity (21 mm) against *F. oxysporum* at 30^{o}C (Fig. 6e). The strain showed minimum growth and activity at 20^{o}C and continued to grow upto 50^{o}C. This could be expected as most of the actinomycetes isolated from soil are mesophilic and grow well between 25-30^{o}C. Similarly, the culture filtrates of antagonistic *Streptomyce*s sp. are reported to have highest antifungal activity against *Helminthosporium oryzae* and *F. solani* at 30^{o}C[29].

The changes in the initial pH of medium affect many cellular processes including regulation and biosynthesis of secondary metabolites. The change in pH also induces the production of new products that adversely affect antibiotic production. The effect of pH on growth and antibiotic production for *S. indiaensis* SRT-1 is presented in the Figure 6f. The pH 8.5 was found to be the optimum for growth (370 mg/100 ml) as well as antifungal metabolite production (19 mm) for isolate. The extremes of acidic and alkaline pH did not support antibiotic production and the growth was also not much appreciable. This is expected as actinomycetes are intolerant to acidity and their numbers decline at or below pH 5. The most conducive range of pH for actinomycetes is between 6.5 and 8.0[30] and in most published literature optimum pH for antibiotic production in *Streptomyces* cultures has been reported to be near neutral[31,23]. As shown in Figure 6g, *S. indiaensis* SRT-1 grew well and produced detectable amounts of antibiotic in presence of 1 - 6% NaCl and the maximum growth and inhibitory effect of isolate was found when grown at 2% salinity. The growth and activity was absent at higher concentrations (14-20%) indicating sensitivity of isolate to salt stress. Similarly, Vijayakumar *et al*[23] reported maximum inhibitory effect of *Streptomyces* sp. VPTS3-1 against *Bacillus subtilis* and *Proteus mirabilis* at 4% salinity and no antimicrobial activity at 32%.

To determine the potency of antifungal antibiotic, it was necessary to establish the nature of bioactive compound and hence MIC and MFC values were determined. Based on these values, it was recognized that *Fusarium oxysporum* NCIM 1072 was most sensitive to the antibiotic produced by *S. indiaensis* as compared to other test fungi (Table 6). The MIC and MFC values for test fungi were in the range of 20 - 40 µg/ml and 30 - 60 µg/ml, respectively. The MIC and MFC values are not constant for a given organisms and it varies with nature of antimicrobial agent, growth conditions, inoculum size and

Table 6 — MIC and MFC values of the antibiotic from *S. indiaensis* SRT-1 against test organisms

S. No	Test organism	MIC (µg/ml)	MFC (µg/ml)
1	*U. maydis* (NCIM 983)	40	50
2	*R. oryzae* (NCIM 878)	40	60
3	*M. hiemelis* (NCIM 873)	30	60
4	*H. graminum* (NCIM 1070)	40	50
5	*T. rubrum* (MTCC 296)	40	60
6	*F. oxysporum* (NCIM 1072)	20	30
7	*A. fumigatus* (MTCC 2544)	30	40
8	*A. flavus* (NCIM 1028)	30	40
9	*A. niger* (NCIM 586)	30	40
10	*A. solani*	40	60

concentration of extract. In our previous studies, we reported MIC of *Azotobacter vinelandii* antifungal metabolite against *F. oxysporum* to be 10 µg/ml[32]. However, the MIC values of antimicrobial agents reported for different strains of *F. oxysporum* were 100 µg/ml[33] and 12.8 µg/ml[34].

The significant reduction in conidial germination and impaired mycelial growth of *F. oxysporum* was observed in presence of antifungal metabolite. The extract inhibited 58% inhibition in spore germination at 20 µg/ml. The delayed spore germination, appearance of segmented mycelium, ruptured and distorted spores indicated the inhibitory action of metabolite on spore germination and mycelial development. Similarly, *in vitro* tests with metabolites from *Streptomyces pulcher* and *Streptomyces canescens*, demonstrated a significant effect in the reduction of spore germination, mycelium growth and sporulation of tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici*[6]. The fungal spores can act as inoculum as well as main disseminating structures of pathogen; hence inhibition of spore germination by secondary metabolites produced by actinomycetes can be considered an important biocontrol mechanism. Thus, the present study demonstrated the efficacy of the metabolite as a capable antifungal agent.

Considering the need for new, safe and more effective antifungal antibiotics in agricultural research, present study highlighted the bio-technological potential of *Streptomyces indiaensis* SRT-1 which has been reported for the first time to produce structurally novel broad spectrum antifungal metabolite. Our results strongly suggest that Marathwada soils can serve as potent source of novel antifungal compounds.

Acknowledgements

The authors would like to thank the SRTM University, Nanded for providing the facilities needed to conduct this research and we also thank Dr. Ashish Polkade, NCCS, India for extending assistance in identification of isolate.

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