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Full Length Research Paper

Investigation of extracellular antifungal proteinaceous compound produced by *Streptomyces* sp. 5K10

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A *Streptomyces* sp. strain named 5K10, isolated from soil samples of Kaziranga National Park, Assam, India was found to secrete an antimicrobial proteinaceous compound, showing *in vitro* antagonistic effect against some pathogenic bacteria, yeasts, dermatophytes and filamentous fungi. Capacity to produce extracellular antimicrobial substances by the strain 5K10 was assessed by conventional spot inoculation method in agar media against test microorganisms as well as fungal spore germination inhibition assay by microscopic observations. The fermented culture broth with ammonium sulfate precipitation revealed a biologically active proteinaceous compound and molecular weight estimated to be about 14.3 kDa on SDS-PAGE gel. Using *Candida albicans* MTCC 227 as the indicator strain, antagonistic activity of the proteinaceous compound in the region of the SDS-PAGE gel was confirmed. Bioactivity of the extracellular protein was sensitive to proteinases and partially susceptible to prolonged heat treatment. The results suggest that *Streptomyces* sp. 5K10 might be an important bioresource of lead molecules for developing antimicrobial agents to control bacterial and fungal infections.

Key words: Antifungal activity, extracellular protein, dermatophytes, *Streptomyces*.

INTRODUCTION

Fungi are an extremely diverse group of organisms, with about 250,000 species widely distributed in essentially every ecosystem. Humans and other animals are exposed to fungi from the moment of birth. Fortunately, only 150-200 or so species are pathogenic to humans and mammals, although many non-pathogenic fungi cause allergy symptoms (Chakrabarti, 2005). During the last few decades, the incidence of human systemic fungal infections, especially involving immunocompromised patients, has dramatically increased. Up to 7% patients dying in teaching hospitals have invasive aspergillosis (Vogesar et al., 1997). *Candida* spp. accounts for 8-15% of nosocomial blood stream infections and is the fourth most common isolate of patients of intensive care unit (Pfaller and Diekema, 2002). Also, skin infection due to dermatophytes has become a significant health problem in countries like India and may reach epidemic proportions in areas with high rate of humidity, over population and poor hygienic conditions (Madhavi et al., 2011). Dermatophytes are fungi that can cause

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superficial infections of the skin, hair and nails. They are the most common agents of fungal infections worldwide and impact millions of individuals annually (Weitzman and Summerbell, 1995; Hainer, 2003). *Trichophyton rubrum* is the most commonly observed dermatophyte worldwide and especially dominant in onychomycosis (Evans, 1998). Only a limited number of antifungal agents are currently available for the treatment of fungal infections (Vicente et al., 2003) and the search for a new, safer, broad-spectrum antifungal antibiotic with greater potency has been progressing slowly (Gupte et al., 2002). Antifungal proteins that are ubiquitous in nature are suitable fungicidal agents because of their natural origin and reduced side effects (Lay and Anderson, 2005; Zhang et al., 2008). Proteins derived from the imperfect filamentous fungi *Aspergillus* and *Penicillium* have broad spectrum antifungal properties (Gunlee et al., 1999; Skouri-Gargouri and Gargouri, 2008). Therefore, the need for new, safe and more effective antifungal agents, preferably naturally occurring with novel mechanisms of action, is a major challenge to the pharmaceutical industry today, especially with the increase in opportunistic infections in the immunocompromised host.

Actinomycetes, especially *Streptomyces* spp., have the exceptional ability to produce a broad range of low molecular weight antibiotics and other secondary metabolites; many of these compounds have antibacterial and antifungal properties and are used as therapeutic agents in medicine and agriculture. The species belonging to the genus *Streptomyces* constitute 50% of the total population of soil actinomycetes and 75-80% of the commercially and medicinally useful antibiotics have been derived from this genus (Mellouli et al., 2003). Furthermore, *Streptomyces* spp. are also well known for their capacity to produce a great variety of secreted proteins, including hydrolytic enzymes that degrade organic material in the soil, such as chitin, cellulose, xylan and starch (Peczynska-Czoch and Modarski, 1988), and enzyme inhibitors (Doran et al., 1990; Goto et al., 1983). In our continued search for novel microbial metabolites having agricultural and pharmaceutical potential, a number of *Streptomyces* strains were screened from forest soil of North East India. This has resulted in isolation of a promising strain designated as *Streptomyces* sp. 5K10 which produced in the culture broth, a bioactive compound endowed with promising antifungal activity. In the present communication, in order to study which products were involved in the antifungal property of the strain, we detected an extracellular bioactive proteinaceous substance secreted by *Streptomyces* sp. 5K10.

MATERIALS AND METHODS

Microorganisms and culture conditions

Streptomyces strain 5K10 was isolated from a soil sample of the Kaziranga National Park (26°30 N to 26°45 N and 93°08 E to 93°36 E), Assam, India. The *Streptomyces* strain is maintained in Streptomyces agar (HiMedia, Mumbai) at 4°C during two months, and in a freezer at -80°C in the presence of glycerol (15%, v/v) for a longer period. The target strains used for screening antimicrobial activity were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India and are: *Staphylococcus aureus* MTCC 737, *Bacillus subtilis* MTCC 441, *Proteus vulgaris* MTCC 426, *Escherichia coli* MTCC 443, *Pseudomonas aeruginosa* MTCC 741, *Candida albicans* MTCC 227, *Saccharomyces cerevisiae* MTCC 170, *Fusarium oxysporum* MTCC 284, *Fusarium moniliforme* MTCC 156, *Aspergillus niger* MTCC 282, *Trichophyton rubrum* MTCC 8477, *Trichophyton mentagrophytes* MTCC 8476 and *Trichophyton tonsurans* MTCC 8475. All bacterial and fungal strains were maintained at 4°C and grown on media recommended by MTCC.

In vitro screening of antimicrobial activity

Production of extracellular antimicrobial substances by the strain 5K10 was preliminary assessed by conventional spot inoculation method (Shomurat et al., 1979) on actinomycetes isolation agar medium (HiMedia, Mumbai). *Candida albicans* and test bacterial strains were grown overnight on sabouraud dextrose broth and nutrient broth (HiMedia, Mumbai) respectively. The fungal strains were grown on potato dextrose agar medium for 5-7 days at 25°C. For determination of the antimicrobial activity of *Streptomyces* sp. 5K10, the inoculated plates were incubated at 28°C for six days, and then inverted for 40 min over chloroform in fumehood. Fungal conidia from agar medium were harvested in sterile distilled water and diluted to 10^5 spores/ml, bacterial and yeast suspensions were diluted to 10^7 CFU (colony forming units)/ml in 0.6% agar medium. *Streptomyces* sp. 5K10 culture on agar plates were then covered with a thin layer of 0.6% agar medium previously inoculated with one of the test organisms. Standard antibiotic gentamicin (10 µg/disc) and fluconazole (10 µg/disc) were procured from HiMedia, Mumbai and used in disc diffusion assay as positive control for bacterial and fungal test organisms, respectively. The antimicrobial activity was observed after 24 h incubation at 37°C for bacteria and 48 h incubation at 28°C for fungi and yeast. The inhibiting activity of precipitated protein suspension of the *Streptomyces* sp. 5K10 was further investigated only against test microbial strains that were sensitive in spot inoculation assay.

Streptomyces sp. 5K10 was grown in yeast-malt-extract medium, consisting of (g/l) yeast extract, 4.0; malt extract, 10.0 and glycerol, 4.0, pH 7.5 and incubated in a rotary shaker at 200 rpm and 28°C for seven days. After harvesting of the mycelia by centrifugation, the extracellular protein fraction of the supernatant (1 L) was precipitated with 70% ammonium sulfate. The precipitated protein collected by centrifugation (18,000 ×g, 30 min, 4°C) was dissolved in 5 ml of 20 mM Tris-HCl/OH buffer, pH 7.5 and dialysed against the same buffer at 4°C for 24 h; finally it was sterilized by filtration through a 47 mm cellulose nitrate membrane, pore size 0.22 µ (HiMedia, Mumbai). Protein concentration in the dialysed preparation was 10.6 mg protein/ml, determined by the method described by Bradford (1976), using bovine serum albumin as a standard. The antifungal activity of the resulting precipitate was measured using the fungal spore germination inhibition assay and the agar well diffusion assay on plates pre-seeded with the indicator strain.

Screening for chitinaseproduction

Screening for chitinase production of *Streptomyces* sp. 5K10 was done by plate agar assay. Colloidal chitin was prepared as described by Khan et al. (2010). The practical grade chitin powder (HiMedia, Mumbai) was used to prepare the colloidal chitin. Chitin

powder (40 g) was dissolved in 500 ml of concentrated hydrochloric acid and continuously stirred at 4°C for 1 h. After stirring, the hydrolyzed chitin was washed a number of times with distilled water in order to remove the acid completely and hence bring the pH to the range of 6-7. As the desired pH was attained, the colloidal chitin was filtered through Whatman filter paper No. 1. The sieved colloidal chitin was subsequently collected and stored in the form of a paste at 4°C. This colloidal chitin was used as the sole carbon source with other minimal salts and agar. The colloidal chitin medium contained (g/l): colloidal chitin, 15; yeast extract, 0.5; $(NH_4)_2SO_4$, 1; $MgSO_4 \cdot 6H_2O$, 0.3; KH_2PO_4, 1.36 and agar, 20; pH 7.5 (Rugthaworn et al., 2007). The plates were incubated for 15 days at 28°C.

Investigation of antifungal property of precipitated protein

SDS-PAGE and direct inhibition assay

The dialysed protein suspension was processed for gel electrophoresis (SDS-PAGE) on 15% polyacrylamide gels (Maniatis et al., 1982). After electrophoresis, the gel was divided into two parts. To detect antimicrobial activity *in situ*, the first half of the gel was soaked in a solution composed of 20% isopropanol and 10% acetic acid for 2 h to remove the SDS, followed by rinsing in sterile water for 4 h (Fulgueira et al., 2004). The washed gels were placed on a sterile Petri plate, overlaid with soft Sabouraud Dextrose Agar (0.8%), containing *C. albicans* MTCC 227 (10^7 CFU/ml). The plate-gel combination was incubated at 28°C for 48 h prior to examination for zones of clearing over the bands (Bhunia et al., 1987). Proteins were visualized after staining the second half of the gel with Coomassie Blue R 250. The molecular mass of the purified antifungal protein was determined by comparison of its electrophoretic mobility with those of molecular mass marker proteins.

Fungal spore germination inhibition assay

One-hundred microliters of spore (10^5 spores/ml) suspension of *A. niger* MTCC 282 previously prepared were added to precipitated protein suspension (25-200 µg protein) in potato dextrose broth. SterileTris-HCl/OH buffer (20 mM, pH 6.8) was used as a control. 100 µl of the mixture was added into a sterile hollow-ground slide with a cover and then incubated in the dark at 25°C in humidified Petri dishes with three replicates. The suspension was observed under a microscope at 40x magnification (Model: Motic BA-410) after 0, 48 and 72 h of incubation, and the percentage conidia germination was determined. The inhibiting activity was estimated as percentage of relative activity (Ar%). Ar% inhibiting activity of germination of fungal conidia was calculated as: $[Gc - G)\ Gc^{-1}] \times 100$, where Gc is the control germination percentage and G is the germination percentage in the presence of protein sample (0-200 µg/ml) in potato dextrose broth as described by Fulgueira et al. (2004).

Effect of pH, temperature and enzymes on antifungal properties of precipitated protein

The pH- and temperature-dependence of the antifungal activity of the precipitated protein suspension was investigated against *A. niger* MTCC 282 in potato dextrose agar medium using agar well diffusion method (Grammer, 1976). The inocula 100 µl of spore (10^5 spores/ml) suspension was uniformly and aseptically spread on the potato dextrose agar surface. The precipitated protein sample (200 µg diluted in 20 mMTris-HCl/OH buffers at different pH range from pH 5.0 to 9.0 were used in the pH-dependence investigations. Same amount of precipitated protein sample (200 µg protein, diluted in 20 mM Tris-HCl/OH buffer, pH 8.5) treated at different temperatures (from 25 to 100°C for 20 min) were used for the temperature-dependence investigations. Sterile buffers without protein were used as negative controls in these experiments. 100 µl of the precipitated protein solution was filled into the agar wells. Plates were kept at 4°C for at least 30 min to allow the diffusion of protein solution. The diameters of the inhibition zones were documented after incubation for 48, 72 and 96 h at 25°C.

Stability toward proteases was assayed by incubating 200 µl (400 µg) of precipitated protein suspension of *Streptomyces* sp. 5K10 and stock solutions of the enzymes elastase (20 µg/ml final concentration at 37°C for 2 h), pronase E (20 µg/ml final concentration at 37°C for 1 h), and proteinase K (10 µg/ml final concentration at 37°C for 1 h). The inhibiting activity of the samples was determined by using the described agar diffusion inhibition assay with *C. albicans* MTCC 227 and *A. niger* MTCC 282 as the test organisms. All the experiments were repeated at least three times to ascertain the activity of test samples.

Statistical analysis

The significance of differences in test fungal spore germination inhibitory activity, effect of different pH conditions and heat treatment on antifungal activity of precipitated protein was determined by analysis of variance (ANOVA), Students' *t*-test. The significance level for all analysis was $p < 0.001$-0.05.

RESULTS AND DISCUSSION

In vitro antimicrobial activity

The strain *Streptomyces* sp. 5K10 showed *in vitro* antimicrobial activity against test bacteria, pathogenic fungi and dermatophytes with maximum inhibitory effect against *C. albicans* (Figures 1 and 2). The strain displayed antifungal activity against all the test fungi in agar medium except phytopathogen *F. oxysporum* (Figure 2). Among all the filamentous fungal test pathogens, maximum mean value of inhibition zones (mm ± SD) was recorded with *A. niger* (40.33 ± 0.58) followed by *T. tonsurans* (33.33 ± 1.53), *F. moniliforme* (33 ± 1.00), *T. mentagrophytes* (31 ± 1.00) and *T. rubrum* (30 ± 1.00) (Figure 2). The strain showed inhibitory activity against Gram-positive bacteria but displayed selective inhibition against Gram-negative bacteria. This strain had no effect on the growth of *P. aeruginosa*. However, it inhibited the growth of *P. vulgaris* and *E. coli* (Figure 2).

The result is in agreement with the findings of Valanarasu et al. (2010) who reported the isolation of a promising *Streptomyces* strain ERI-04 from forest soil sample and showed strong broad-spectrum antifungal activity against fungi and dermatophytes (Valanarasu et al., 2010). Over 50 different antibacterial agents including streptomycin, neomycin, chloramphenicol and tetracyclines in addition to many antifungal agents like macrolide polyenes (nystatin amphotericin and natamycin) were derived from *Streptomyces* species (Raja and Prabakarana, 2011).

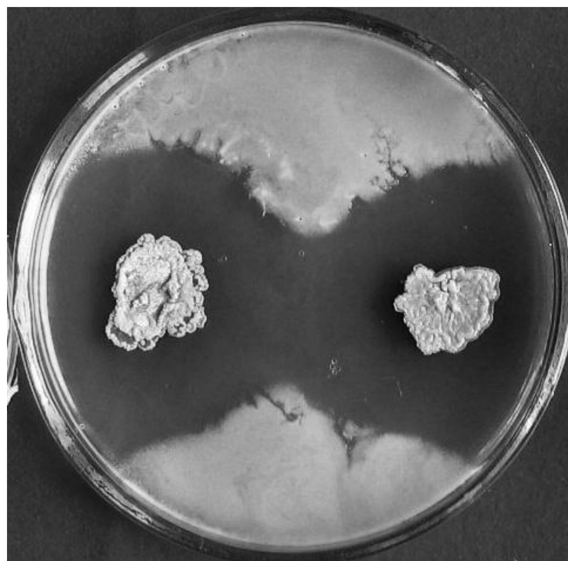

Figure 1. Inhibitory activity of *Streptomyces* sp. 5K10 against *C. albicans* MTCC 227 by spot inoculation method.

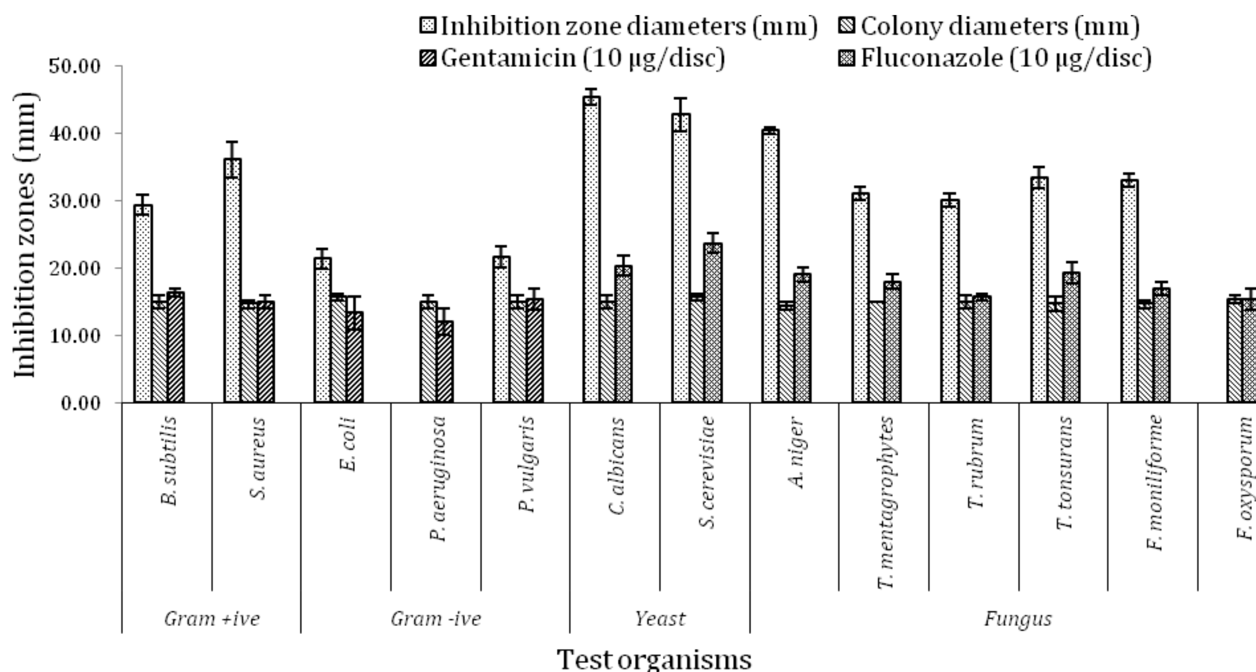

Figure 2. *In vitro* antimicrobial activity of *Streptomyces* sp. 5K10 against test organisms in agar medium. Each value is the mean of three replicates (N=3) and *error bar at* each point indicated that ± SD.

Fungal conidia germination inhibition

Five pathogenic fungal strains were further tested for conidia germination inhibition in the presence of precipitated protein (25-200 µg) recovered from the culture broth of the *Streptomyces* sp. 5K10 (Table 1). Fungal conidia germination inhibition percentage of relative activity (Ar%) was maximum in *T. tonsurans* (80.20 ± 0.30%) followed by *F. moniliforme* (78.63 ± 71%), *T. rubrum* (75.57 ±0.55%), *T. mentagrophytes* (74.75 ± 1.22%) *and A. niger* (70.21 ± 0.79%) after 48 h of incubation in the presence of 200 µg protein sample

Table 1. Fungal conidia germination inhibitory activity in the presence of precipitated protein recovered from the culture broth of the *Streptomyces* sp. 5K10.

Test organism	Time (h)	Germination inhibitory activity (Ar%)[a] Precipitated protein (µg)			
		25	50	100	200
F. moniliforme	48	21.15 (± 0.30)	42.01 (± 0.58)	54.72 (± 1.03)	78.63 (± 0.71)
MTCC 156	72	24.21 (± 0.71)	50.65 (± 0.43)	63.59 (± 0.84)	87.28 (± 0.57)
A. niger	48	32.21 (± 0.71)	42.90 (± 0.75)	56.90 (± 0.36)	70.21 (± 0.79)
MTCC 282	72	43.58 (± 0.81)	75.14 (± 1.06)	84.24 (± 1.23)	98.90 (± 0.36)
T. rubrum	48	20.94 (± 0.17)	37.83 (± 0.24)	51.72 (± 1.03)	75.57 (± 0.55)
MTCC 8477	72	22.77 (± 0.30)	45.81 (± 0.56)	58.88 (± 0.65)	81.49 (± 0.79)
T. mentagrophytes	48	23.89 (± 0.29)	42.07 (± 0.47)	55.22 (± 0.11)	74.75 (± 1.22)
MTCC 8476	72	28.22 (± 0.99)	50.65 (± 0.43)	63.53 (± 0.93)	85.32 (± 0.56)
T. tonsurans	48	27.53 (± 0.52)	48.09 (± 0.12)	64.90 (± 0.82)	80.20 (± 0.30)
MTCC 8475	72	30.60 (± 0.46)	61.67 (± 0.58)	70.20 (± 0.32)	89.72 (± 0.25)

[a]Ar% inhibiting activity of germination of fungal conidia was calculated as: $[Gc - G)\ Gc^{-1}] \times 100$, where Gc is the control germination percentage and G is the germination percentage in the presence of protein sample (0-200 µg) of *Streptomyces* 5K10 in potato dextrose broth. Each value is the mean of three replicates (N=3) and SD was calculated for each experiment indicated in brackets. Significant differences (p-values) were determined based on comparison with the untreated samples (0 µg protein sample, 100% germination) and showed significant differences ($p < 0.001$) for all samples.

(Table 1) .The present observation also suggested that among the test filamentous fungi, *A. niger* was the most sensitive to the precipitated protein (200 µg) after 72 h of incubation (98.90 ± 0.36% germination inhibition) and 25 µg protein sample was still partially inhibitory (32.21 ± 0.71% and 43.58 ± 0.81% germination inhibition after 48 and 72 h of incubation, respectively) (Table 1). Germination inhibition of precipitated protein was maintained when the test tubes were further incubated at room temperature for 96 h, making evident the inhibitory effect of this compound (data not shown). An antifungal protein PPEBL21 is also reported from *E. coli* BL21 strain to have an inhibitory effect on growth of pathogenic fungi. This protein inhibited growth of *A. fumigates* and *A. flavus* in microbroth dilution and percentage spore germination inhibition assays (Yadav et al., 2007).

It is common to find the production of exoenzyme with chitinolytic activityin *Streptomyces* spp. (Fulgueira et al., 2004). Many species of chitinolytic actinomycetes, especially those belonging to the genus *Streptomyces* exhibit antagonism towards phytopathogenic fungi (Nagpure and Gupta, 2013). Therefore, colloidal chitin degradation test was done with the strain *Streptomyces* sp.5K10 and it was found that the strain did not degrade colloidal chitin after incubation for up to 15 days.

The results obtained in the present study indicated that the antagonistic effect was due to the production of compounds with fungicidal and not chitinolytic activity. Previous study demonstrated that the antagonistic effect of *Streptomyces* sp. C/33-6 was due to the production of compounds with fungicidal and not chitinolytic activity against toxigenic fungi (Fulgueira et al., 2004). In our study, extracellular antifungal metabolite produced by the strain *Streptomyces* sp. 5K10 showed similar features. This proteinaceous compound isolated from the culture broth of the strain *Streptomyces* sp. 5K10 has characteristics similar to killer toxin (Fulgueira et al., 2004; Ohki et al., 2001).

Characterization of the biologically active compound

SDS-PAGE and direct inhibition assay

An attempt was made to determine the molecular mass of biologically active protein present in the culture filtrates of *Streptomyces* sp. 5K10 on polyacrylamide gel. SDS-PAGE gels containing the dialysedprotein samples were stained with Coomasie blue R 250 (Figure 3). Gel revealed a major peptide band along with few other bands, and this zone containing the specific band was localized only by its biological activity when overlaid with *C. albicans* MTCC 227 in 0.7% PDA medium (Figure 3). The molecular mass of the proteinaceous compound was estimated from this gel to be about 14.3 kDa.

Antifungal proteins and polypeptides have been isolated from diverse groups of organisms, including plants, bacteria, fungi and insects (Iijima et al., 1993; Caruso et al., 1996; Bormann et al., 1999; Hao et al., 1999). Although microorganisms belonging to the *Actinomycetes* class are known to produce a variety of antibiotics and extracellular enzymes, there have been few reports on antifungal proteins such as the SKLP produced by *Streptomyces* sp. F-287 (Hiraga et al., 1999; Ohki et al., 2001), the AFP1 produced by *Streptomyces tendae* (Bormann et al., 1999) and a proteinaceous com-

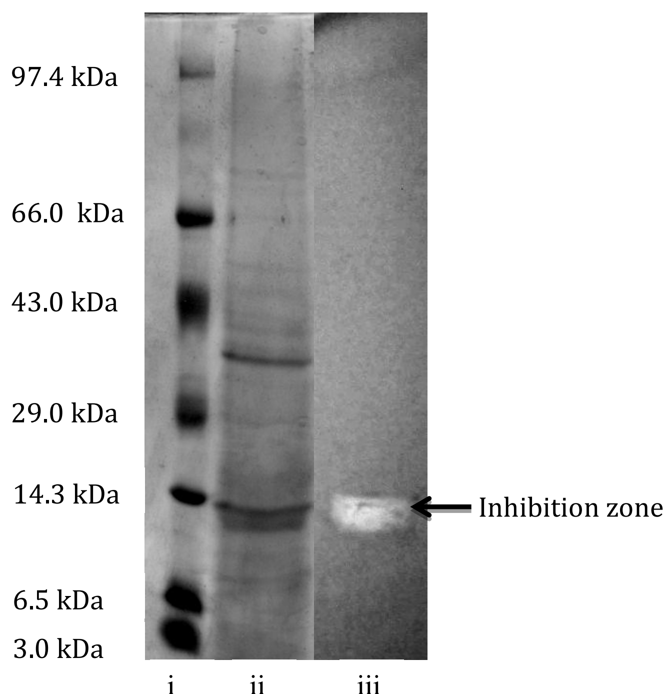

Figure 3. SDS-PAGE analysis and detection of antimicrobial activity. (a) Molecular weight of the antimicrobial compound as estimated by SDS-PAGE, (b) gel overlaid with the indicator strain *C. albicans* MTCC 227. i: Molecular weight standards (mass in kDa). ii: 50 µg proteins were loaded; iii: inhibition zone corresponding to the protein band. The zone of clearing represents the antimicrobial activity of proteinaceous compound.

pound from *Streptomyces* sp. C/33-6 with cytocidal but not chitinolytic activity reported against toxigenic fungi (Fulgueira et al., 2004). Malik et al. (2008)also reported that *Streptomyces fulvissimus* 95-K secreted a high molecular weight peptide of 63 kDa inhibitory to *Micrococcus luteus*, *B. subtilis*, *B. cerus* and methicillin resistant *S. aureus* (MRSA) strains.

When the protein recovered from culture broth was incubated in the presence of elastase, pronase E and proteinase K, the antagonistic activity was not detected against *C. albicans* MTCC 227 and *A. niger* MTCC 282 in agar diffusion assay and antifungal activity in polyacrylamide gel was completely lost (data not shown).

Effect of pH, temperature and enzymes

Effect of different pH and temperature conditions on the biological activity of the biologically active protein was determined by using agar diffusion test against *A. niger* (Tables 2 and 3). Precipitated protein from the culture broth of *Streptomyces* sp. 5K10 showed the highest antifungal activity at pH 8.0 - 9.0, but it was maintained in different rate under all investigated pH conditions (Table2). Antifungal activity was observed after treatment at pH 9.5, although activity progressively degraded between pH 10.0 - 10.5 (data not shown). Precipitated protein after treated at different temperatures for 20 min showed maximum activity against *A. niger* (inhibition zone 23.60 ± 1.00 mm) at 25°C. The protein proved to be stable even after a temperature treatment at 100°C for 20 min, and the antifungal effect of the treated protein was remained after 96 h of incubation (Table 3). Antifungal activity after treatment at 100°C for 30 min was not observed.

The effects of various proteolytic enzymes, that is, elastase, pronase E and proteinase K, on the protein recovered from the culture broth was investigated. The inhibiting activity of the protein samples incubated with proteinases was determined by agar diffusion inhibition assay with *C. albicans* and *A. niger* as the test organism. Biological activity was completely sensitive to all the proteinase tested and growth inhibition zones were not detected.

Conclusion

In the present study, a proteinaceous compound was detected in the culture supernatant of the strain *Streptomyces* sp. 5K10 having both antifungal and antibacterial activity. The extracellular biologically active metabolites produced by the strain showed inhibitory activity against Gram-positive bacteria but displayed selective inhibition against Gram-negative bacteria and phytopathogens in agar diffusion assay. *Streptomyces* sp. 5K10 displayed highest antagonistic activity against *C. albicans* and *S. cerevisiae* in spot inoculation assay. Also, the extracellular metabolites secreted by the strain showed promising inhibitory effects on filamentous fungi, *A. niger* followed by *T. tonsurans*, *F. moniliforme*, *T. mentagrophytes and T. rubrum.*

Precipitated proteinaceous compound recovered from the culture supernatant of the strain after ammonium sulfate precipitation exhibited strong antifungal activity against fungal test pathogens by inhibiting spore germination. The relative molecular weight of bioactive proteinaceous compound was estimated to be around 14.3 kDa on SDS-PAGE using molecular mass marker proteins. Precipitated protein showed the highest anti-fungal activity at pH 8.0 - 9.0 and antifungal activity after treatment at 100°C for 30 min was not observed. Biological activity was not observed after the treatment with selective proteinase.

These results suggest that at least part of the chemical structure of this compound corresponded to a proteinaceous compound. This antifungal protein secreted by *Streptomyces* sp. 5K10 is expected to develop as a biological prevention agent against pathogenic fungi and dermatophytes after purification and characterization. The present findings also indicated that *Streptomyces* could be a source of the secondary metabolites with promising antifungal activity and the importance for further investigation on the goal of obtaining novel antimicrobial agent out of the

Table 2. Effect of different pH conditions on antifungal activity of precipitated protein (200 µg) recovered from the culture broth of the *Streptomyces* sp. 5K10 in agar diffusion test against *A. niger* MTCC 282.

pH	The diameter of inhibitory zones (mm)		
	48 h	72 h	96 h
5	15.67*** (± 0.58)	14.67*** (± 0.58)	13.33*** (± 0.58)
6	16.33** (± 1.15)	15.33** (± 0.58)	14.33*** (± 0.58)
7	18.67*** (± 0.58)	17.00** (± 0.00)	16.33*** (± 0.58)
7.5	20.00** (± 1.00)	18.33** (± 0.58)	17.33*** (± 0.58)
8	22.67^{NS} (± 0.58)	20.33^{NS} (± 1.15)	19.33*** (± 0.58)
8.5	23.67 (± 0.58)	22.67 (± 1.15)	23.00 (± 0.00)
9	22.67^{NS} (± 0.58)	20.00* (± 0.00)	18.00$^{\$}$ (± 0.00)

Inhibition zones represent the mean of three replicates (N=3) and SD was calculated for each experiment indicated in brackets. Significant differences (*p*-values) were determined based on the comparison with the samples treated at the optimal pH (8.5) at 48 h. ^{NS}Not significant; $^{\$}$Could not calculate the'*t*' values as the SD were 0.00 for both groups.*p < 0.05; **p < 0.01; ***p < 0.001.

Table 3. Effect of heat treatment on antifungal activity of precipitated protein (200 µg) recovered from the culture broth of the *Streptomyces* sp. 5K10 in agar diffusion test against *A. niger* MTCC 282.

Temperature (°C)	The diameter of inhibitory zones (mm)		
	48 h	72 h	96 h
40	22.33^{NS} (± 0.58)	21.67^{NS} (± 1.15)	21.33^{NS} (± 0.58)
50	20.00^{NS} (± 1.00)	19.33^{NS} (± 0.58)	19.00^{NS} (± 0.00)
60	20.00^{NS} (± 1.15)	18.67^{NS} (± 1.00)	18.67^{NS} (± 1.00)
80	19.33^{NS} (± 0.58)	18.67^{NS} (± 0.58)	18.33^{NS} (± 0.58)
100	16.33** (± 0.58)	14.33* (± 0.58)	13.67* (± 0.58)
Control (25°C)	23.60 (± 1.00)	22.40 (± 1.73)	21.00 (± 1.73)

Inhibition zones represent the mean of three replicates (N=3) and SD was calculated for each experiment indicated in brackets. Significant differences (*p*-values) were determined based on the comparison with the samples treated at control temperature (25°C). ^{NS}Not significant. *p < 0.05; **p < 0.01.

Actinomycetes from unscreened ecosystem and the least investigated area.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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