Isolation, characterization and optimization of antifungal activity of an actinomycete of soil origin

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About 312 actinomycetes were isolated from soil samples on chitin agar. All these isolates were purified and screened for their antifungal activity against pathogenic fungi. Out of these, 22% of the isolates exhibited activity against fungi. One promising isolate with strong antifungal activity against pathogenic fungi was selected for further studies. This isolate was from Pune, and was active against both yeasts and molds. Various fermentation parameters were optimized. Based on morphological and biochemical parameters, the isolate was identified as Streptomyces. The correlation of antifungal activity with growth indicated growth dependent production of antimetabolite. Maximum antifungal metabolite production (600 units/ml) was achieved in the late log phase, which remained constant during stationary phase, and it was extracellular in nature.

Keywords: Antifungal activity, Screening, Streptomyces sp. PU 23.

Fungi are eukaryotic and have machinery for protein and nucleic acid synthesis similar to that of higher animals. It is, therefore, difficult to find out compounds that selectively inhibit fungal metabolism and exhibiting no toxicity to humans. There is an evidence that some fungal strains are resistant to certain antifungal drugs with resulting therapeutic failures. Therefore, there is a pressing need of non-toxic and effective antifungal antibiotics. The pioneering work of Waksman showed that actinomycetes are capable of producing medically useful antibiotics. Actinomycetes are diverse group of heterotrophic prokaryotes forming hyphae at some stage of their growth, hence referred as filamentous prokaryotes. They are a successful group of bacteria that occur in a multiplicity of natural and man-made environments and a unique group having different morphological, cultural, biochemical and physiological characters. Approaches to search for and discovery of new antibiotics are generally based on screening of naturally occurring actinomycetes. The main objective of the present study was to isolate, screen and characterize naturally occurring antifungal actinomycetes and to optimize the fermentation parameters.

Materials and Methods

Collection of soil samples—Soil samples were collected from three Indian states viz., Maharashtra, Karnataka and Kerala. The samples were collected in sterile containers and maintained at 4°C until analysis.

Isolation and screening of antifungal actinomycetes—Actinomycetes were isolated on chitin agar which were incubated at 37°C for 7 days. All these cultures were purified by streak plate technique and confirmed by colony morphology and screened for their antifungal activity. One promising strain, PU 23, was selected and grown on different agar media viz., starch casein, glucose asparagine, glycerol asparagine, potato dextrose, Sabouraud dextrose and yeast extract malt extract, to have medium that stimulates maximum antifungal activity. After incubation for 7 days at 37°C, agar discs of actinomycete growth were made with a sterile cork borer and placed on Sabouraud dextrose agar plates (pH 5.6) seeded with the fungal culture. The plates were incubated at 28°C and observed for antibiosis after 24 hr in case of yeasts and 96 hr in case of molds. Similarly, the antifungal activity of the culture supernatant of the actinomycete in above said liquid medium was tested by agar well diffusion method.

Characterization of selected actinomycete isolate—The growth of the organism was studied at 22°, 28°, 37° and 42°C temperatures. Morphological features of the actinomycete were studied under phase
Contrast microscope (Nikon, Japan). Same culture was subjected to cultural, physiological and biochemical characterisation. Cultural characterization was done on ISP media viz., yeast extract malt extract agar (ISP-2), oatmeal agar (ISP-3), inorganic salts starch agar (ISP-4), glycerol asparagine agar (ISP-5), peptone yeast extract iron agar (ISP-6), tyrosine agar (ISP-7) and glucose asparagine agar (ISP-5) at 37°C. The substrate and aerial mycelium colour including soluble pigments were assigned using the colour harmony manual (4th edition; 1958; Container Corporation of America; Chicago, Illinois). Utilization of different carbon and nitrogen sources such as D-glucose, D-galactose, D-fructose, D-mannitol, D-xylene, L-arabinose, L-rhamnose, raffinose, sucrose, adonitol, D-L-alanine, L-cysteine, L-histidine, L-lysinol, L-phenylalanine and L-valine was studied. The cells were analysed for 2, 6-diaminopimelic acid and whole cell sugars content.

Optimization of antifungal metabolite production—
Antifungal metabolite production was carried out in 100 ml starch casein medium (starch, 1%; casein, 0.1%; KH₂PO₄, 0.05%; MgSO₄·7H₂O, 0.05%; pH 7) in 500 ml Erlenmeyer flasks. The unit of antifungal activity was arbitrarily defined as the amount corresponding to 0.3 mm (diam.) of inhibition zone of Aspergillus niger under defined conditions.

Temperature—
Five 500 ml Erlenmeyer flasks, each with 100 ml starch casein medium were inoculated with the actinomycete spores at the rate of 1×10⁶ spores ml⁻¹ of production medium. The flasks were incubated at 37°C on shaker at 250 rpm. After every 24 hr, the culture broth was analysed for antifungal metabolite content by well diffusion method and biomass in terms of OD₅₆₀ for 12 days. Besides, the pH was monitored by digital pH meter.

Detection of antifungal metabolite in the cell mass and supernatant—To test if the antifungal metabolite production was intracellular or extracellular, the culture was centrifuged at 10,000 rpm for 20 min. The supernatant and ethanol extract of the dried biomass were tested for their antifungal activity with ethanol as control.

Results
Isolation and screening—In all 321 strains of actinomycetes were isolated and screened against yeasts and molds. Amongst them, 22% showed antifungal activity against fungi. The antifungal activity of the strain PU 23 against the pathogenic fungi was good when grown on starch casein medium indicating that the starch casein agar medium was good for inducing antifungal activity in the strain PU 23 (Fig. 1). In the shake flask study, the culture supernatant of the actinomycete also showed good antifungal activity.

Characterization of actinomycetes isolate—The selected isolate, PU 23, had an optimum temperature for growth at 37°C. It could grow very well on all the ISP media and produce water soluble brown pigment on all the media except oatmeal agar and yeast extract malt extract agar, however, it had yellow colour on reverse on the latter media. The aerial mycelium was grey on all media except tyrosine agar and yeast extract malt extract agar on which it was white. The spore chains were spiral type and each had more than 15 spores per chain (Fig.2). The cell wall of the strain PU 23 contained 1-diaminopimelic acid. The biochemical properties have been shown in Table 1. The taxonomic properties described above apparently suggested that the isolate PU 23 belongs to the genus Streptomyces and designated as Streptomyces sp. PU 23.

Optimization of temperature, pH, agitation and glycerol concentration for antifungal metabolite production—The optimum conditions for antifungal metabolite production were pH 7, temperature 37°C, agitation 250 rpm and glycerol 1.5% (Fig.3) and the activity was equivalent to 600 units/ml.
Fig. 1—Strong antifungal activity exhibited by starch casein grown Streptomyces sp. PU 23 against — Molds: (a) Aspergillus niger NCIM 586 and (b) Fusarium oxysporum NCIM 1072; and Yeasts: (c) Candida albicans NCIM 7102; and (d) Cryptococcus humicolus NRRL 12944.

Fig. 2 — Streptomyces sp. PU 23. a) Spore chains as seen under phase contrast microscope (×400), and b) Growth on starch casein medium showing grey spore mass.
**Time course of antifungal metabolite production in Streptomyces sp. PU 23**—The antifungal metabolite production was monitored over a period of 12 days. The rate of antifungal metabolite production correlated with growth rate of *Streptomyces* PU23 and was highest (600 units/ml) in the late log phase (Fig.4). The pH of the broth was between 6.8 and 7 throughout fermentation.

**Testing for extra- and intra-cellular metabolite production**—The ethanol extract of the biomass did not show antifungal activity. However, the culture supernatant inhibited all fungi tested (Table 2). Therefore, the antifungal metabolite was extracellular in nature.

**Discussion**

There are reports regarding bioactivity amongst chitinolytic actinomycetes. Further, we also propose based on our findings that chitinolytic actinomycetes exhibit promising antifungal activity. The antifungal activity has been observed both in solid as well as in culture broth unlike fumaramycin, which is inactivated in the fermentation broth. Production of antifungal metabolite has been known to be influenced by components of medium and cultural conditions, such as aeration, agitation, pH, temperature and glycerol concentration, which often vary from organism to organism. Cultural conditions such as pH, temperature, aeration and agitation were found to affect antifungal metabolite production by *Streptomyces* sp. PU 23. The optimum pH for metabolite production was found to be 7. It has been reported that the change in pH of the culture medium induces production of new products that adversely affect antibiotic production. It has been reported that the production of helvoic acid and cerulenin by *Cephalosporium caerules* is affected by change in the pH. In *Streptomyces* sp. PU 23, the optimum temperature for metabolite production and growth is same i.e. 37°C. Deviation from this temperature affects the yield of the antifungal metabolite. Agitation affects aeration and mixing of the nutrients in the fermentation medium. The optimum rate of agitation (250 rpm) was found to facilitate increased antifungal metabolite production in the broth.

**Table 2**—MIC values* of the supernatant** from *Streptomyces* sp. PU 23 against fungi in comparison with that of nystatin

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<thead>
<tr>
<th>Target organisms</th>
<th>MIC (units/ml)</th>
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<tr>
<td></td>
<td>Supernatant</td>
</tr>
<tr>
<td><em>Aspergillus niger</em> NCIM 586</td>
<td>1.8</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em> NCIM 1072</td>
<td>1.8</td>
</tr>
<tr>
<td><em>Candida albicans</em> NCIM 7102</td>
<td>3.75</td>
</tr>
<tr>
<td><em>Cryptococcus humicola</em> NRRL 12944</td>
<td>3.75</td>
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* Determined by broth tube dilution procedure using two fold dilution in Sabaroud dextrose broth at 28°C.
** Inoculum (1×10⁶ spores ml⁻¹) in 100 ml starch casein broth in 500 ml Erlenmeyer flask, incubated on rotary shaker (250 rpm) for 7 days at 37°C, followed by centrifugation to obtain supernatant.

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**Fig. 3**—Conditions required for optimum production of antimetabolite

**Fig. 4**—Time course of antimetabolite production by *Streptomyces* sp. PU 23. [●]—Antimetabolite produced (units/ml); and [■]—O.D. at 540 nm.
present study, which might be due to better transfer of oxygen and enhanced uptake of nutrients. It has been reported that the yield of cephalosporin C increases with increase in dissolved oxygen.

Rate of production of the antifungal metabolite was directly proportional to growth rate. Maximum metabolite production in the stationary phase as antibiotic production in the log phase, which remained constant during stationary phase. However, there are many reports about the antibiotic production in the stationary phase as antibiotic is a secondary metabolite. The antifungal metabolite of the strain was extracellular in nature. In most of the cases, antibiotics are extracellular. Further studies on the extraction, purification and characterization of the antifungal metabolite are currently in progress.

References