Production of a growth dependent metabolite active against dermatophytes by *Streptomyces rochei* AK 39

S.K. Augustine, S.P. Bhavsar & B.P. Kapadnis

Department of Microbiology, University of Pune, Pune, India

Received March 26, 2004

**Background & objectives:** Dermatophytes responsible for causing dermatophytoses in humans have acquired resistance to certain antimycotic drugs. We isolated naturally occurring actinomycetes with an ability to produce metabolites having antimycotic property. The time-course of antifungal metabolite production in terms of arbitrary units (AU) under optimum conditions was studied.

**Methods:** Water and soil samples were collected from various locations. The actinomycetes were isolated on starch casein medium and screened for their antifungal activity against yeasts and molds including dermatophytes. One promising isolate which showed a unique, stable and interesting property of inhibiting only dermatophytes was selected and characterized. Optimization of antifungal metabolite production in terms of AU using *Trichophyton rubrum* as target was done. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of the culture supernatant from the isolate and that of griseofulvin were determined for all dermatophytes.

**Results:** Of the 218 actinomycete isolates, 14 per cent produced the metabolites having antifungal activity. The selected actinomycete, identified as *Streptomyces rochei* AK 39 produced metabolite, which was active against only dermatophytes whereas yeasts and other molds were resistant to it. Starch casein medium was found to be good for inducing antifungal activity in the isolate. The maximum antifungal metabolite production (400 AU/ml) was achieved in the late log phase, which remained constant during the stationery phase, and it was extracellular in nature. The MIC and MFC values of the culture supernatant from the isolate against the dermatophytes were within the range 1.25 to 5 and 1.25 to 10 AU/ml respectively.

**Interpretation & conclusion:** The metabolite from *Streptomyces rochei* AK 39 was produced during late log phase and was active against only dermatophytes with a greater potency than griseofulvin. However, this needs further investigation using purified powdered form of the active component.

**Key words** Antifungal - arbitrary unit - dermatophytes - screening - *Streptomyces rochei* AK 39

Fungi are eukaryotic and have machinery for protein and nucleic acid synthesis similar to that of higher animals. It is, therefore, very difficult to find out compounds that selectively inhibit fungal metabolism without exhibiting any toxicity to humans\(^1\). Dermatophytes are a group of fungi...
responsible for causing dermatophytoses in humans and there is evidence that the dermatophytes have acquired resistance to certain antimycotic drugs. As there is lack of effective and safe antifungal antibiotics, there is a need of nontoxic 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 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 the search for and discovery of new antibiotics are generally based on screening of naturally occurring actinomycetes. The objective of the present study was to isolate naturally occurring actinomycetes with an ability to produce metabolites having antimycotic property. During our initial survey of actinomycetes producing metabolites having antimycotic property, one particular isolate had a unique property of inhibiting only dermatophytes and this property was very stable. Since there are no reports of metabolites from *Streptomyces rochei* inhibiting dermatophytes, it was chosen for further studies.

**Material & Methods**

Collection of soil samples: Water (n=14) and soil (n=12) samples were collected from different locations in and around Pune, Maharashtra, India in sterile containers and maintained at 4°C until analysis.

Isolation and screening of antifungal actinomycetes: Actinomycetes were isolated on starch casein medium by serial dilution method. All cultures were purified by streak plate technique and confirmed by colony morphology and screened for their antifungal activity. One promising isolate, *Streptomyces rochei* AK 39, 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 know which medium stimulates maximum antifungal activity. All media were obtained from Hi-Media, Mumbai. 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 (SDA) plates (pH 5.6) seeded with the fungal culture. The plates were incubated at 28°C and observed for antibiosis after 24 h in case of yeasts and 96 h in case of molds. The fungal cultures used as targets were *Candida albicans* NCIM 7102, *Cryptococcus humicolus* NRRL 12944, *Aspergillus niger* NCIM 586, *Fusarium oxysporum* NCIM 1072, *Epidermophyton floccosum* MTCC 613, *Trichophyton rubrum* MTCC 296, *Trichophyton mentagrophytes* BJMC and *Microsporum gypseum* MTCC 2830 (NCIM: National Collection of Industrial Microorganisms, NRRL: Northern Regional Research Laboratory, MTCC: Microbial Type Culture Collection, BJMC: Byramjee Jeejeebhoy Medical College, Pune). The antifungal activity of the culture supernatant of the actinomycete in above mentioned liquid media was tested by agar well diffusion method.

Characterization of the selected actinomycete isolate: Morphological features of the actinomycete were studied by inserting sterile coverslips in the solid starch casein medium at an angle of 45°. Spore suspension of the actinomycete was inoculated at the intersection of the medium and coverslip. The coverslips were observed under phase contrast microscope (Nikon, Japan) after incubation for one week at 28°C. Cultural characterization was done on ISP (International *Streptomyces* Project) 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) and tyrosine agar (ISP- 7) at 37°C. All media were obtained from Hi-Media, Mumbai. The growth of the organism was studied at different temperatures viz., 22, 28, 37 and 42°C. Utilization of different carbon and nitrogen sources such as D-glucose, D-galactose, D-fructose, D-mannitol, D-xylose, L- arabinose, L- rhamnose, L-cysteine, L-histidine, L-leucine, L-phenylalanine and L-valine was studied. Chemotaxonomic studies were done by analyzing the cells for 2, 6 - diaminopimelic acid and whole cell sugar content. Using the above mentioned data on characterization, the probability matrix for the actinomycete isolate was calculated using the probabilistic identification of bacteria (PIB) software and identified up to species level.
Production of antifungal metabolite: Spore suspension of *Streptomyces rochei* AK 39 was inoculated into 100 ml starch casein broth (10^6 spores/ml of the medium) in 500 ml Erlenmeyer flask. The flask was incubated on shaker (250 rpm) for 7 days at 37°C. The culture was centrifuged at 5, 500 g for 20 min. The culture supernatant was used as a source of antifungal metabolite.

Slope of inhibition zone versus concentration of test using *Trichophyton rubrum* as target culture: Two-fold dilutions of the culture supernatant were made in sterile distilled water. 100 µl aliquot of each dilution was introduced into agar wells (10 mm) in SDA plates (pH 5.6) seeded with the spore suspension (10^6 spores/ml) of *Trichophyton rubrum*. These plates were incubated at 28°C for 96 h. The inhibition zone diameters were recorded and plotted against concentration of the culture supernatant. The data were subjected to regression analysis in order to determine the slope, which indicates increase in inhibition zone for two-fold increase in dose of antifungal metabolite. The slope was designated as arbitrary unit (AU)\(^{13}\).

Optimization of antifungal metabolite production: The yield of the antifungal metabolite was monitored in terms of arbitrary units (AU). Antifungal metabolite production was carried out in 100 ml starch casein medium (starch 1%, casein 0.1%, KH\(_2\)PO\(_4\) 0.05%, MgSO\(_4\).7H\(_2\)O 0.05%, pH- 7) in 500 ml Erlenmeyer flasks.

Temperature: Five 500 ml Erlenmeyer flasks, each with 100 ml starch casein medium were inoculated with the actinomycete spores at the rate of 1x10^6 spores/ml of the medium. These flasks were incubated at different temperatures \(v\text{iz.}, 24, 28, 32, 37\) and 42°C on rotary shaker (Steelmet, India) for 7 days.

\(\text{pH}\): The initial \(\text{pH}\) of the starch casein fermentation medium was adjusted to 4, 5, 6, 7, 8 and 9 separately with 0.1N NaOH/0.1N HCl. All flasks were inoculated as mentioned above and incubated at 37°C on rotary shaker (200 rpm) for 7 days.

Agitation: Five 500 ml Erlenmeyer flasks, each with 100 ml starch casein medium were inoculated as mentioned above and incubated on rotary shaker at 100, 150, 200, 250 and 300 rpm for 7 days at 37°C.

Glycerol concentration: Effect of glycerol at varied concentrations \(v\text{iz.}, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5\) and 1.6 per cent (v/v) was studied on antifungal metabolite production. The inoculum size and incubation conditions were the same as mentioned earlier.

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of the supernatant from *Streptomyces rochei* AK 39 against dermatophytes: The MIC values of the culture supernatant and an antimycotic agent griseofulvin were determined by broth tube dilution procedure using two-fold dilution in Sabouraud dextrose medium at 28°C, incubated for 96 h\(^9\). The potency of griseofulvin was 200,000 units/ml. The 100 µl aliquot of each dilution was tested against *Trichophyton rubrum*. MFC of culture supernatant and griseofulvin was determined by subculturing 50 µl from the tubes not visibly turbid and spot inoculating on SDA plates\(^14\). MFCs were determined as the lowest concentration resulting in no growth on subculture\(^15\).

Time course of antifungal metabolite production under optimum conditions: The 500 ml Erlenmeyer flask with 100 ml starch casein broth was inoculated with spores at the rate of 1x 10^6 spores/ml of production medium. The flasks were incubated at 37°C on shaker at 250 rpm. After every 24 h, the culture broth was analysed for antifungal metabolite content by well diffusion method and biomass in terms of OD\(_{540}\) for 12 days\(^10\). Besides, the \(\text{pH}\) of the broth was monitored by digital \(\text{pH}\) meter (LabIndia Instruments, Thane).

Detection of antifungal metabolite in the cell mass and supernatant: To test if the antifungal metabolite production is intracellular or extracellular, the culture was centrifuged at 5,500 g for 20 min. The supernatant and biomass were extracted with ethyl acetate and ethanol respectively and tested for their antifungal activity using the agar well diffusion method\(^10\). Ethyl acetate and ethanol were used as control.
Results

Isolation and screening of antifungal actinomycetes: Of the 218 actinomycete isolates obtained from 26 soil and water samples, 31 (14%) produced the metabolites having antifungal activity. The isolate, AK 39, exhibited strong antifungal activity against the dermatophytes when grown on starch casein media indicating that the secondary metabolite was produced in optimum amount on starch casein agar medium. The metabolite from the isolate was active against only dermatophytes whereas yeasts (Candida albicans and Cryptococcus humicolus) and other molds (Aspergillus niger and Fusarium oxysporum) were resistant. In the shake flask study, the culture supernatant of the actinomycete also showed antifungal activity against dermatophytes (Table I).

Characterization and identification of actinomycete isolate: The selected isolate, AK 39, had an optimum temperature for growth at 37°C. It could grow well on all the ISP media and produced water soluble brown pigment. The aerial mycelium was grey on all media. The spore chains were spiral type and each had more than 12 spores per chain. The isolate could utilize all the carbon and nitrogen sources except L-arabinose, D-xylose and L-valine. The cell wall of the strain contained 2, 6- diaminopimelic acid. Based on these data, the probability matrix for the isolate was calculated using the software and was found to be 0.98, which is close to Streptomyces rochei, and according to the software the present isolate confirms to Streptomyces rochei. The strain was designated as Streptomyces rochei AK 39.

Slope of inhibition zone versus concentration of test using Trichophyton rubrum as target culture: The yield of the antifungal metabolite was expressed in terms of arbitrary units. There was a linearity between inhibition zone diameter and the concentration of the culture supernatant. Therefore, the slope was determined by regression analysis of the data. The slope was found to be 0.5, designated in arbitrary unit (AU). The yield and potency of the test antifungal were expressed in terms of AUs. Thus 1 AU corresponds to increase in inhibition zone by 0.5 mm corresponding to double dilution using Trichophyton rubrum as target organism under defined assay conditions.

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 200 rpm and glycerol 1.2 per cent and the activity was equivalent to 400 AU/ml.

MIC and MFC values of the supernatant from Streptomyces rochei AK 39 against dermatophytes: The MIC and MFC values of the supernatant were lower as compared to griseofulvin for all the dermatophytes tested (Table II). However, this needs further study using purified powdered form of the active component.

Time course of antifungal metabolite production in Streptomyces rochei AK 39: The antifungal metabolite production was monitored over a period of 12 days. The rate of antifungal metabolite production correlated with growth rate of the Streptomyces rochei AK 39.
and was highest (400 AU/ml) in the late log phase (Fig.). The pH of the broth was within the range 6.6 to 7 throughout fermentation.

**Testing for extra-and intracellular metabolite production:** The ethanol extract of the biomass did not show antifungal activity. However, the ethyl acetate extract of the culture supernatant inhibited all dermatophytes tested showing that the antifungal metabolite was extracellular in nature.

**Discussion**

The antibacterial\(^\text{16}\) and enzymatic\(^\text{17}\) activities of *Streptomyces rochei* are well known, however its antifungal property has been reported only against *Aspergillus fumigatus\(^\text{18}\)*. *Streptomyces rochei* AK 39 was active against dermatophytes whereas yeasts and other molds were resistant. The supernatant from starch casein grown *Streptomyces rochei* AK 39 showed greater potency against the dermatophytes than griseofulvin. However, this needs further investigation using purified powdered form of the active component.

In our earlier studies we observed that RPMI 1640 medium buffered with morpholinepropanesulfonic acid (MOPS) as proposed by the National Committee for Clinical Laboratory Standards (NCCLS) does not adequately support the growth of the target cultures and the incubation time required is too long\(^\text{19}\). Similar problems were earlier reported\(^\text{20}\). Hence MIC was

---

**Table I. Activity of *Streptomyces rochei* AK 39 grown on different media against dermatophytes**

<table>
<thead>
<tr>
<th><em>Streptomyces rochei</em> AK 39 grown on</th>
<th>Zone diameter of inhibition (mm) by</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Agar disc</strong></td>
<td><strong>Supernatant</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Ef</strong></td>
<td><strong>Tr</strong></td>
</tr>
<tr>
<td>Starch casein</td>
<td>22</td>
<td>34</td>
</tr>
<tr>
<td>Yeast extract-malt extract</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Sabouraud dextrose</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Glycerol asparagine</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Glucose asparagine</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Potato dextrose</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

Zone diameter of inhibition excludes the size of the well (10 mm)

Ef: *Epidermophyton floccosum* MTCC 613, Tr: *Trichophyton rubrum* MTCC 296

Tm: *Trichophyton mentagrophytes* BJMC, Mg: *Microsporum gypseum* MTCC 2830

**Table II. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of the culture supernatant of *Streptomyces rochei* AK 39, and griseofulvin against dermatophytes**

<table>
<thead>
<tr>
<th>Test organisms</th>
<th>Culture supernatant</th>
<th>Griseofulvin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (AU/ml)</td>
<td>MFC (AU/ml)</td>
</tr>
<tr>
<td><strong>Epidermophyton floccosum</strong></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td><strong>Trichophyton rubrum</strong></td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td><strong>Trichophyton mentagrophytes</strong></td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td><strong>Microsporum gypseum</strong></td>
<td>2.5</td>
<td>5</td>
</tr>
</tbody>
</table>

|                                     | MIC (units/ml)      | MFC (units/ml) |
|**Epidermophyton floccosum**         | 6                   | 12            |
|**Trichophyton rubrum**              | 3                   | 6             |
|**Trichophyton mentagrophytes**      | 3                   | 6             |
|**Microsporum gypseum**              | 6                   | 12            |

The yeasts, *Candida albicans* and *Cryptococcus humicolus* and other molds, *Aspergillus niger* and *Fusarium oxysporum*, were resistant.
determined by broth tube dilution procedure using two-fold dilutions of metabolite in Sabouraud dextrose broth\(^9\). The antifungal activity was seen both on solid as well as in culture broth unlike fumaramidmycin, which is inactivated in the fermentation broth\(^{21}\). Production of antifungal metabolite has been known to be influenced by media components and cultural conditions, such as aeration, agitation, \(pH\), temperature and glycerol concentration, which vary from organism to organism\(^{22}\). Cultural conditions were found to affect antifungal metabolite production by \textit{Streptomyces rochei} AK 39. The change in \(pH\) of the culture medium induces production of new substances that affect antibiotic production\(^{23}\). The production of helvoic acid and cerulenin by \textit{Cephalosporium caeruleus} was affected by change in the \(pH\)\(^{24}\). In \textit{Streptomyces rochei} AK 39, the optimum temperature for metabolite production and growth was the same \textit{i.e.}, 37\(^\circ\)C. Deviation from optimum temperature for antifungal metabolite production severely affects the yield of the antifungal metabolite\(^{25}\). Agitation affects aeration and mixing of the nutrients in the fermentation medium. Adequate agitation (200 rpm) was found to increase antifungal metabolite production in the present study. The yield of cephalosporin C is known to be increased with increased agitation\(^{26}\).

Maximum production of metabolite (400 AU/ml) was achieved in late log phase, which remained constant during stationery phase. Antibiotic production usually occurs in the stationery phase\(^{27}\). In our case, the production of the metabolite by \textit{Streptomyces rochei} AK 39 took place during the late log phase of growth in the fermentation medium indicating that the metabolite production was directly proportional to the growth rate. Accumulation of antibiotics, cephalomycin C and clavulanic acid has been shown to occur in parallel with growth in a defined medium\(^{28}\). The antifungal metabolite was active in solid as well as liquid media and it was extracted by ethyl acetate from the culture supernatant, whereas the ethanol extract of the biomass showed no antifungal activity, thus showing the extracellular nature of the metabolite. Mostly antibiotics are extracellular\(^{29}\). Further studies on the extraction, purification and characterization of the antifungal metabolite are currently in progress.

In conclusion, the findings of the present study showed that naturally occurring actinomycetes have a great potential to produce metabolites against dermatophytes enabling the discovery of new antibiotics and hence merit future studies.

**Acknowledgment**

The authors thank the Head, Department of Microbiology, University of Pune for providing the laboratory facilities.

**References**


Reprint requests: Dr B.P. Kapadnis, Professor, Department of Microbiology, University of Pune Ganeshkhind, Pune 411007, India e-mail: bpkapadnis@yahoo.com