CHAPTER 5

CHARACTERIZATION OF THE ANTIBIOTIC FROM *Streptomyces* sp. PU 23
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*Streptomyces* sp. PU 23

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5.1 Introduction

Actinomycetes have been described as the greatest source of antibiotics since Waksman introduced actinomycetes into his systematic screening programme for new antibiotics in the early 1940s. They have provided about two-thirds (more than 4000) of the naturally occurring antibiotics discovered, including many of those important in medicine, such as aminoglycosides, anthracyclines, chloramphenicol, β-lactams, macrolides, tetracyclines etc. 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 toxicity to humans (Glazer and Nikaido, 1995).

During our survey of actinomycetes for their antifungal activity, we investigated the fermentation product of *Streptomyces* sp. PU 23 isolated from the Pune University campus soil. The fermentation parameters are described in the previous chapter. This antibiotic showed strong antifungal activity with minimum inhibitory concentration (MIC) values between 20 to 40 μg/ml. By using elemental analysis, UV (ultra violet), FTIR (Fourier Transform Infrared), NMR (Nuclear magnetic resonance, 500 MHz), DEPT (Distortionless Enhancement by Polarization Transfer, 500 MHz) and LC-Mass spectra, the compound was predicted to be a straight chain polyhydroxy polyether compound with a double bond.

Ultraviolet spectroscopy is primarily used to measure the multiple bond or aromatic conjugation within molecules. The UV region extends from 1000 - 4000 Å or 100 - 400 nanometers (nm). The UV measurements are reported in nm. The vacuum UV below 200 nm is so named because the molecules of air absorb radiation in this region, and thus this region is accessible only with special vacuum equipment. The ultraviolet spectrum records the wavelength of an absorption maximum, i.e., λ_max and the strength of the absorption, i.e., molar absorptivity. On passing electromagnetic radiation in the ultraviolet and visible regions through a compound with multiple bonds, a portion of the radiation is normally absorbed by the compound. The amount of the absorption depends on the wavelength of the radiation and structure of the compound. The absorption of radiation is due to the subtraction of energy from the radiation beam.
when electrons in orbitals of lower energy excite into orbitals of higher energy. Since
this is an electron excitation phenomenon, UV is sometimes called electronic
spectroscopy (Kalsi, 2002; Kemp, 1967).

The infrared spectroscopy (IR) is one of the most widely used tools for the
detection of functional groups in pure compounds and mixtures and for compound
comparison. The spectrum is obtained in minutes using a few mg of the compound
which can also be recovered. It is one of the most powerful analytical techniques, which
offers the possibility of chemical identification. This technique when coupled with
intensity measurements may be used for quantitative analysis. One of the most
important advantages of infrared spectroscopy over the other usual methods of
structural analysis (X-ray diffraction, electron spin resonance, etc.) is that it provides
useful information about the structure of molecule quickly, without tiresome evaluation
methods. The infrared data advantageously complement the results obtained by other
methods. The technique is based upon the simple fact that a chemical substance shows
marked selective absorption in the infrared region. After absorption of IR radiations, the
molecules of a chemical substance vibrate at many rates of vibration, giving rise to
close packed absorption bands, called an IR absorption spectrum, which may extend
over a wide wavelength range. Various bands will be present in IR spectrum, which will
correspond to the characteristic functional groups and bonds present in a chemical
substance. Band positions in an infrared spectrum may be expressed conveniently by
the wave number \( \nu \), whose unit is cm\(^{-1}\). Thus, an IR spectrum of a chemical substance is
a fingerprint for its identification (Chatwal and Anand, 2000).

Nuclear magnetic resonance (NMR) spectroscopy, as is implied in the name,
involves the change of the spin state of a nuclear magnetic moment when the nucleus
absorbs electromagnetic radiation in a strong magnetic field. NMR is a branch of
spectroscopy in which radio frequency waves induce transitions between magnetic
energy levels of nuclei of a molecule (Kalsi, 2002; Kemp, 1967). In NMR, a molecular
sample, usually dissolved in a solvent, is placed in a magnetic field and the absorption
of radio-frequency waves by certain nuclei (protons and others) is measured. Two types
of NMR spectroscopy in common use today are notably \(^1\text{H}\) (proton, i.e., PNMR) and
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$^{13}$C (carbon-13, i.e., CNMR). It is a powerful tool for investigating nuclear structure (Boyer, 2001).

Mass spectrometry is used to characterize organic molecules in two principal ways: (1) to measure exact molecular weights, and from this, exact molecular formulae can be determined, (2) to indicate within a molecule the points at which it prefers to fragment; from this, the presence of certain structural units in the organic compound can be recognized (Kalisi, 2002; Kemp, 1967). In the technique of mass spectrometry, the compound under investigation is bombarded with a beam of electrons which produce an ionic molecule or ionic fragments of the original sample. The resulting assortment of charged particles is then separated according to their masses. The spectrum produced, known as mass spectrum, is a record of information regarding various masses produced and their relative abundance. It is an analytical technique, which can provide information concerning the molecular structure and can be used to determine directly molecular weight as high as 4000. It is one of the few methods that can be used as a qualitative analytical tool to characterize different organic substances. The mass spectrometer is becoming a common tool mainly because of its speed, reliability and ability to give the largest amount of specific information about the substances to be analyzed (Chatwal and Anand, 2000).

Further studies on the shelf life of the antibiotic from *Streptomyces* sp. PU 23 and effect of temperature and pH were carried out. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of the antibiotic against different target organisms were determined (Cappuccino and Sherman, 1999; Hammer et al, 2002). Effect of detergents and enzymes on activity of the antibiotic against the target organisms was studied (Munimbazi and Bullerman, 1998).

In order to determine the effect of the antibiotic on the ergosterol present in the fungal cell membrane, ergosterol was used as the reversal agent to test for its ability to reverse the inhibition of the target organisms caused by the antibiotic (Kalantar, 2003). Thus the antibiotic from *Streptomyces* sp. PU 23 is a straight chain polyhydroxy polyether compound and probably binds to the ergosterol present in the fungal cell.
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membrane resulting in the leakage of intracellular material and eventually death of the cell.

5.2 Materials and methods
5.2.1 Structural analysis of the antibiotic
5.2.1.1 Elemental analysis and melting point of the compound

The elemental analysis was carried out in the micro analytical laboratory of the National Chemical Laboratory, Pune 411008, India. The analysis was done using CHNS analyzer (Vario-El, Germany). Melting point was determined on electrically heated oil bath (Thomas Hoover, U.S.A.).

5.2.1.2 UV and IR spectra

Ultraviolet spectra were recorded on Shimadzu UV-170 spectrophotometer. One mg of sample was dissolved in 10 ml water and the spectra were recorded at 200-400 nm range. The infrared spectra were recorded on Shimadzu IR-470 model. The spectra were scanned in the 400 to 4000 cm⁻¹ range. The spectra were obtained using potassium bromide pellet technique. Potassium bromide (AR grade) was dried under vacuum at 100°C for 48 hours and 100 mg of KBr with 1 mg of sample was taken to prepare KBr pellet. The spectra were plotted as intensity versus wavenumber.

5.2.1.3 NMR and LCMS spectra

The pure antibiotic sample was subjected to ¹HNMR, ¹³CNMR and DEPT spectra (500 MHz, Brucker Biospin, Switzerland) The antibiotic sample was dissolved (3mg for ¹HNMR and 10 mg for ¹³CNMR) in 3 ml of D₂O and analyzed by NMR with DSS (2, 2 –dimethyl – 2 – silapentane –5 – sulfonate ) as internal standard. The LCMS spectrum was obtained from HP CHEM instrument (GC-LC/MS).
5.2.2 Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of the antibiotic from *Streptomyces* sp. PU 23 against different target organisms

The MIC and MFC values of the antibiotic were determined by broth tube dilution procedure using two fold dilution in Sabouraud dextrose broth at 28°C (Cappuccino and Sherman, 1999). The MFC values of the antibiotic were determined by subculturing 50 µl from tubes not visibly turbid and spot inoculating onto Sabouraud dextrose agar (SDA) plates. MFC values were determined as the lowest concentration that prevented growth on subculture (Lavermicocca et al., 2003; Hammer et al., 2002).

5.2.3 Determination of shelf life of the antibiotic from *Streptomyces* sp. PU 23

The effect of storage time on antifungal activity of the antibiotic (100 µg/ml) was determined by storing the antibiotic at 4°C in different ampoules for 1 hour, 24 hours, 1 week, 1 month, 2 months, 3 months, 6 months and 12 months. After the specified storage period, 100 µl from each tube was added in wells prepared in Sabouraud dextrose agar plates already seeded with the target organisms i.e.* Aspergillus niger* and *Candida albicans* separately. The wells were prepared by using a sterile cork borer of 10 mm diameter. The results were recorded after incubation at 28°C for four days.

5.2.4 Thermal stability of the antibiotic from *Streptomyces* sp. PU 23

To determine the effect of temperature on stability of the antibiotic, screw capped ampoules, each with 100 µg/ml of the antibiotic in water were kept at temperatures 30, 40, 50, 60, 70 and 80°C for one hour in water bath. The antibiotic solutions were cooled to room temperature and volumes were brought to the original and the residual antifungal activity was determined against the target cultures as described under 5.2.3. The antibiotic was also subjected to autoclaving temperature (121°C for 15 minutes).
5.2.5 Effect of pH on activity and stability of the antibiotic from *Streptomyces* sp. PU 23

To determine the effect of pH on stability of the antibiotic, 100 µg of the antibiotic was mixed with 1ml of 0.1N phosphate buffer of varied pH in various tubes, incubated for one hour at 30°C and the residual antifungal activity in each tube was determined against both *Aspergillus niger* and *Candida albicans* as target organisms as described under 5.2.3.

5.2.6 Synergistic effect of the antibiotic from *Streptomyces* sp. PU 23 with hamycin

The antagonistic activity of the antibiotic from *Streptomyces* sp. PU 23 in combination with hamycin was tested against *Candida albicans*. Whatman paper No.1 strips (10 × 45 mm) each containing 100µg of the antibiotic from *Streptomyces* sp. PU 23 and strips containing 100 units of hamycin per strip were prepared and placed perpendicular to each other on the agar plates already spread inoculated with *C.albicans* and incubated at 28°C for two days. The inhibition was observed in presence and absence of hamycin (Cappuccino and Sherman, 1999).

5.2.6 Effect of ergosterol on antifungal activity of the antibiotic from *Streptomyces* sp. PU 23

In order to determine the effect of the antibiotic from *Streptomyces* sp.PU 23 on the ergosterol present in the fungal cell membrane, ergosterol was used as the reversal agent to test for its ability to reverse the inhibition of *C. albicans* caused by the antibiotic from *Streptomyces* sp. PU 23. Sabouraud dextrose agar plates with 0.5% ergosterol was prepared along with a control without ergosterol. The plates were seeded with the test organism. Wells were made with a sterile cork borer and 0.1ml of the antibiotic (100 µg/ml) was added to the well. The plates were incubated at 28°C for 24 hours and observed for the zone of inhibition (Kalantar, 2003).
5.2.8 Effect of detergents on activity of the antibiotic from *Streptomyces* sp.PU 23 against *C. albicans*

Susceptibility of the antibiotic to denaturation by various detergents, viz; Tween 20, Tween 80, Triton X-100, Sodium dodecyl sulphate (SDS) and Cetrimide was determined by mixing the detergents with the antibiotic and incubating them at 30°C for six hours. Detergents were dissolved in distilled water at concentration of 0.01g/ml. 100 µl of the antibiotic solution (100 µg/ml) was mixed with 100 µl of detergent and incubated as mentioned above (Munimbazi and Bullerman, 1998). Detergents added to distilled water were used as controls to check the effect of detergents themselves on *C. albicans*. Activity was tested as described under 5.2.3.

5.2.9 Effect of enzymes on activity of the antibiotic from *Streptomyces* sp.PU 23 against *A. niger* and *C. albicans*

The sensitivity of the antibiotic to denaturation by enzymes Proteinase K, Trypsin, Lipase and Lysozyme was tested. All the enzymes were obtained from Sigma Chemical Co. and were dissolved in distilled water at concentration 1 mg/ml. 100 µl of the antibiotic solution (100 µg/ml) was mixed with 100 µl enzyme and incubated at 30°C for 3 hours. The antibiotic solution without any enzymes served as control (Munimbazi and Bullerman, 1998). The residual antifungal activity of the mixture was tested against *A. niger* and *C. albicans* by the agar well diffusion method.

5.3 Results

5.3.1 Elemental analysis and melting point of the compound

The elemental analysis showed the presence of carbon 37.09% and hydrogen 6.42%. The antibiotic is a brown coloured, amorphous powder having a melting point of 218-220°C.

5.3.2 Structural analysis of the antibiotic.

The UV (ultra violet) spectral data exhibited strong absorption at λ<sub>max</sub> 211, 216 and 218nm, suggesting a carbon-carbon double bond. The FTIR (Fourier Transform
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Infrared spectrum exhibited absorption bands at 3296 and 1031.8 cm⁻¹, which indicates hydroxyl groups, and at 1639 cm⁻¹ indicating a double bond. ¹H NMR (Nuclear magnetic resonance, 500 MHz) spectra of the antibiotic in D₂O has peaks in the region 2.7 to 5.4 δ and major peaks between 3.3 and 4.4 δ which probably indicates –CHOH protons. The ¹³C NMR (500 MHz) and DEPT (Distortionless Enhancement by Polarization Transfer, 500 MHz) spectra showed only one –CH₂ group at 63 ppm and two olefinic carbons at 102.241 and 102.354 ppm. The LC-Mass spectrum showed a molecular ion at m/z 528 indicating that the molecular weight is probably 528.

With the above spectral data, the compound probably has a molecular formula of C₁₆H₃₂O₁₉ having one –CH₂OH (hydroxymethyl) group, one olefinic double bond and the rest being –CHOH– (hydroxymethylene), which are linked to each other by ether linkages. Thus the antibiotic probably is a straight chain polyhydroxy polyether compound with a single double bond, indicating a non-polyene antifungal antibiotic.

5.3.3 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of the antibiotic from Streptomyces sp. PU 23 against different target organisms

The MIC and MFC values of the antibiotic were determined by broth tube dilution procedure using two fold dilution in Sabouraud dextrose broth at 28°C. Aspergillus spp. were most sensitive to the antibiotic produced by Streptomyces sp. PU 23 followed by other molds and yeasts as seen by the MIC and MFC values (Table 5.2).
### Table 5.1 Physico-chemical properties of the antibiotic from *Streptomyces* sp. PU 23.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour</td>
<td>Brown</td>
</tr>
<tr>
<td>Nature</td>
<td>Amorphous</td>
</tr>
<tr>
<td>Melting point</td>
<td>218-220°C</td>
</tr>
<tr>
<td>Yield (mg/lit)</td>
<td>1.66</td>
</tr>
<tr>
<td>Rf value</td>
<td>0.78</td>
</tr>
<tr>
<td>Solubility</td>
<td>Water soluble</td>
</tr>
<tr>
<td>Elemental analysis (%)</td>
<td>Carbon 37.09, Hydrogen 6.42</td>
</tr>
<tr>
<td>UV $\lambda_{\text{max}}$ (nm)</td>
<td>211, 216, 218</td>
</tr>
<tr>
<td>IR (KBr) cm$^{-1}$</td>
<td>1031.8, 1639, 3296</td>
</tr>
<tr>
<td>LC-MS (m/z)</td>
<td>528</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>$C_{16}H_{32}O_{19}$</td>
</tr>
</tbody>
</table>
Table 5.2 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of the antibiotic from *Streptomyces* sp. PU 23 against different target organisms*

<table>
<thead>
<tr>
<th>Target organism</th>
<th>MIC (µg/ml)</th>
<th>MFC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus niger</em></td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td><em>Penicillium</em> sp.</td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td><em>Cryptococcus</em> sp.</td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td><em>Cryptococcus humicolus</em></td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Activity was performed by using the agar well diffusion technique
Each data point represents average of three replicates.
The antibiotic was not active against bacteria.
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Figure 5.1 Flow chart for isolation, purification and identification of antibiotic from *Streptomyces* sp. PU 23

1. Fermented starch casein broth
2. Centrifuged to remove the mycelium
3. Supernatant extracted with n-hexane
4. Solvent concentrated in Rota evaporator (40 °C at 50 rpm)
5. Dark brown gummy substance obtained
6. Dissolved in ethanol
7. Solvent concentrated in Rota evaporator (40 °C at 50 rpm)
8. Brown coloured crude antibiotic obtained
10. Silica gel column chromatography
11. Eluted with ethanol: water (50:50) solvent system
12. Fractions collected and checked for antifungal activity.
13. Active fraction separated (Rf: 0.78)
14. Characterization of the antibiotic using elemental analysis, UV, IR, $^1$H NMR, $^{13}$C NMR, DEPT and Mass spectral data.
15. A non-polyene antifungal antibiotic
   (Straight chain polyhydroxy polyether compound with a single double bond)
Fig 5.2 UV- spectrum of the antibiotic from *Streptomyces* sp. PU 23
Fig 5.3 IR- spectrum of the antibiotic from *Streptomyces* sp. PU 23
Fig 5.6 DEPT- spectrum of the antibiotic from *Streptomyces* sp. PU 23
5.3.4 Determination of shelf life of the antibiotic from *Streptomyces* sp. PU 23

To determine the shelf life of the antibiotic, the antibiotic (100 μg/ml) was stored at 4°C in different ampoules for 1 hour, 24 hours, 1 week, 1 month, 2 months, 3 months, 6 months and 12 months. After the specified storage period, the antifungal activity of the antibiotic was determined by well diffusion method. The antibiotic was fairly stable for a period of 12 months at 4°C (Table 5.3).

**Table 5.3 Shelf life of antibiotic from *Streptomyces* sp. PU 23 at 4°C**

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Aspergillus niger</th>
<th>Candida albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hour</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>24 hours</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>1 week</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>1 month</td>
<td>21</td>
<td>16</td>
</tr>
<tr>
<td>2 months</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>3 months</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>6 months</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>12 months</td>
<td>20</td>
<td>15</td>
</tr>
</tbody>
</table>

Each data point represents average of three replicates.
5.3.5 Thermal stability of the antibiotic

Since the activity of the antibiotic was quite stable at 4°C, experiments were also conducted to see the effect of elevated temperatures on stability of the antibiotic. For this purpose the antibiotic was kept at various temperatures (30 to 80°C) for one hour. As seen in table 5.4 the antibiotic was stable at different temperatures, however the antibiotic lost its antifungal activity completely after autoclaving at 121°C for 15 minutes.

Table 5.4 Thermal stability* of the antibiotic from *Streptomyces* sp. PU 23

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th><em>Aspergillus niger</em></th>
<th><em>Candida albicans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>40</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>50</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>60</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>70</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>80</td>
<td>22</td>
<td>16</td>
</tr>
</tbody>
</table>

*The antibiotic lost its activity completely after autoclaving at 121°C for 15 minutes
Each data point represents average of three replicates.
5.3.6 Effect of pH on activity and stability of the antibiotic from *Streptomyces* sp. PU 23

Stability of the antibiotic at different pH was checked by pre-incubating the antibiotic, for one hour, in phosphate buffer at different pH ranging from 5.7-8.0. The antibiotic was quite stable within this pH range as tested against both *Aspergillus niger* and *Candida albicans*. After incubation of the antibiotic at pH in the range of 5.7-5.8, maximum residual activity was observed at pH range 6.8-8, whereas the activity decreased at pH 6.3 and below (Table 5.5).

<table>
<thead>
<tr>
<th>pH</th>
<th>Inhibition zone diameter (mm) against</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Aspergillus niger</em></td>
</tr>
<tr>
<td>5.7</td>
<td>19</td>
</tr>
<tr>
<td>6.3</td>
<td>20</td>
</tr>
<tr>
<td>6.8</td>
<td>22</td>
</tr>
<tr>
<td>7.3</td>
<td>22</td>
</tr>
<tr>
<td>7.8</td>
<td>22</td>
</tr>
<tr>
<td>8</td>
<td>22</td>
</tr>
</tbody>
</table>

Each data point represents average of three replicates.
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5.3.7 Antagonistic activity of the antibiotic from Streptomyces sp. PU 23 in presence of hamycin against C. albicans

The strip containing hamycin (100 unit/strip) and the another one containing the antibiotic from Streptomyces sp. PU 23 (100μg/strip) were kept at right angles on the agar plate, seeded with C. albicans. The inhibition zone was 16 mm along the length of the strip containing the antibiotic (100μg/strip) and was 24 mm along the length of hamycin strip (100 unit/strip); however, at the intersection, widths of inhibition zone increased along both the strips, thus indicating the synergistic effect of the antibiotic from Streptomyces sp. PU 23 with hamycin. The antifungal effect of each drug is enhanced when the two drugs are used in combination.

5.3.8 Effect of ergosterol on antifungal activity of the antibiotic from Streptomyces sp. PU 23

To investigate whether the antibiotic had any affinity towards the ergosterol present in the fungal cell membrane, ergosterol was used as a reversal agent. The control plate without ergosterol showed an inhibition zone diameter of 16 mm, whereas the plate containing the reversal agent, ergosterol, showed a reduced inhibition zone diameter i.e. 7 mm. Thus the antibiotic from Streptomyces sp. PU 23 probably binds to the ergosterol present in the fungal cell membrane resulting in the leakage of intracellular material and eventually death of the cell.

5.3.9 Effect of detergents on activity of the antibiotic from Streptomyces sp. PU 23 against C. albicans

Effect of various detergents, viz; Tween 20, Tween 80, Triton X-100, Sodium dodecyl sulphate (SDS) and Cetrimide on activity of the antibiotic was determined by mixing them with the antibiotic and incubating for six hours at 30°C. The activity was checked against C. albicans. Detergents added to distilled water were used as controls to check the effect of detergents themselves on C. albicans. The antibiotic alone showed 16 mm inhibition zone diameter against C. albicans, while inhibition zone diameter obtained with mixture of antibiotic and detergents ranged between 15 to 17 mm. This
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clearly indicated that there was no significant loss of antifungal activity of the antibiotic after treatment with detergents (Table 5.6).

5.3.10 Effect of enzymes on activity of the antibiotic from *Streptomyces* sp. PU 23 against *A. niger* and *C. albicans*

Sensitivity of the antibiotic produced by *Streptomyces* sp. PU 23 to enzymes like Proteinase K, Trypsin, Lipase and Lysozyme was evaluated. The antibiotic was mixed with respective enzyme and incubated for 3 hours and the activity of the antibiotic was assayed against *A. niger* and *C. albicans*. The antibiotic alone showed 22 mm and 16 mm inhibition zone diameter against *A. niger* and *C. albicans* respectively, while inhibition zone diameter obtained with mixture of antibiotic and enzymes ranged between 21 to 22 mm in case of *A. niger* and 16 to 17 mm in case of *C. albicans*. This clearly indicated that there was no significant loss of antifungal activity of the antibiotic after treatment with the enzymes (Table 5.7).
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Table 5.6 Effect of detergents on activity of the antibiotic from *Streptomyces* sp. PU 23 against *C.albicans*

<table>
<thead>
<tr>
<th>Detergents</th>
<th>Inhibition zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without antibiotic</td>
</tr>
<tr>
<td>Tween 20</td>
<td>0.0</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.0</td>
</tr>
<tr>
<td>Triton X 100</td>
<td>0.0</td>
</tr>
<tr>
<td>SDS</td>
<td>0.0</td>
</tr>
<tr>
<td>Cetrimide</td>
<td>8</td>
</tr>
<tr>
<td>Antibiotic*</td>
<td>-</td>
</tr>
</tbody>
</table>

* Antibiotic concentration 100μg/ml

Each data point is the mean of three experiments.

Table 5.7 Effect of enzymes on activity of the antibiotic from *Streptomyces* sp. PU 23 against *A.niger* and *C.albicans*

<table>
<thead>
<tr>
<th>Test</th>
<th>Inhibition zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>A.niger</em></td>
</tr>
<tr>
<td>Proteinase K + antibiotic</td>
<td>22</td>
</tr>
<tr>
<td>Trypsin + antibiotic</td>
<td>21</td>
</tr>
<tr>
<td>Lipase + antibiotic</td>
<td>22</td>
</tr>
<tr>
<td>Lysozyme + antibiotic</td>
<td>22</td>
</tr>
<tr>
<td>Antibiotic*</td>
<td>22</td>
</tr>
</tbody>
</table>

*Antibiotic alone at concentration 100μg/ml

Each data point is the mean of three experiments.
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5.4 Discussion

*Streptomyces* sp. PU 23 produced an antibiotic showing good activity against different fungal test cultures. This compound was isolated after fermentation of the *Streptomyces* sp. PU 23 in starch casein broth at 37°C for 7 days. The yield was 1.66 mg/lit and was fairly stable for a period of 12 months at 4°C. The antibiotic produced by *Streptomyces* sp. PU 23 was very heat stable and active over a wide range of pH. The antibiotic was kept at various temperatures (30 to 80°C) for one hour and was found to be stable at different temperatures (Table 5.4), however the antibiotic lost its antifungal activity completely after autoclaving at 121°C for 15 minutes. Similar observations on the antifungal metabolites produced by *Bacillus* species stable at various temperatures and active under both acidic pH and basic pH have been reported (Phae *et al.*, 1990; Lebbadi *et al.*, 1994; Motta and Brandelli, 2002; Singh and Garg, 2003).

From our studies we observed that RPMI 1640 medium buffered with 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. Similar problems were earlier reported (Petrou and Shanson, 2000). Hence MIC was determined by broth tube dilution procedure using two fold dilutions of antibiotic in Sabouraud dextrose broth (Collins *et al.*, 1995). It was important to establish whether the antibiotic was fungistatic as well as fungicidal, as an indication of the potential usefulness of the antibiotic in the antifungal treatment. Most isolates showed a difference of concentration between inhibitory and cidal values, indicating that although the antibiotic has fungicidal activity, at particular concentration it is fungistatic only. The MFC values were determined as per Hammer *et al* (2002). There was no significant loss of antifungal activity of the antibiotic after treatment with various detergents and enzymes (Table 5.6 and 5.7).

According to our investigations, the antibiotic from *Streptomyces* sp. PU 23 probably binds to the ergosterol present in the fungal cell membrane resulting in the leakage of intracellular material and eventually death of the cell. This is similar to the mode of action of nystatin and amphotericin B which interact with ergosterol,
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disrupting the fungal membranes (Igraham and Igraham, 2000; Prescott et al., 2002). The antifungal effect of the antibiotic from *Streptomyces* sp.PU 23 and hamycin is enhanced when the two drugs are used in combination against *C. albicans*. Kalantar (2003) had made similar observation by using the antimicrobial metabolite from *Aureobasidium pullulans* and hamycin in combination against yeasts.

On this background, the biotechnological potential of *Streptomyces* sp. PU 23 in terms of production of antibiotic inhibiting pathogenic fungi, both yeasts and molds is noteworthy. Various studies done and results obtained in the present investigation indicated that *Streptomyces* sp. PU 23 produced a non-proteinic, stable antifungal antibiotic which is non polyene in nature and seems to be novel as it does not fit into any of the known classes of antifungal antibiotics.
5.5 References


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