Fungi are eukaryotic and have machinery for protein and nucleic acid synthesis similar to that of animals and plants. It is, therefore, very difficult to find out compounds that selectively inhibit fungal metabolism and are nontoxic to humans. There is an evidence that some fungal strains are resistant to certain antimycotic drugs with resulting therapeutic failures. There is lack of effective and safe antifungal antibiotics. Even today, amphotericin B, a polyene, is the drug of choice to treat systemic fungal infections. However, amphotericin B is toxic to humans and therapy has side effects like renal failure, fever, chills and hypertension. Imidazole inhibits fungal growth by inhibiting sterol biosynthesis but is fungistatic rather than fungicidal. Resistance to azoles is becoming an increasing problem as their use has been increased. Other antifungal agents available are limited by either a narrow spectrum of activity, toxicity or both. Thus very few antibiotics have found utility in therapy. Therefore, there is a pressing 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 to as filamentous prokaryotes. They are gram positive and a successful group of bacteria that occur in a multiplicity of natural and man-made environments. They are a unique group of organisms having different morphological, cultural, biochemical and physiological characters. This group is a potential producer of many enzymes, enzyme inhibitors, growth promoting substances and antibiotics. The actinomycetes have been described as the greatest source of antibiotics. Approaches to the search for and discovery of new antibiotics are generally based on screening of naturally occurring actinomycetes.

In view of this background, the present investigation was undertaken with the following objectives
- Isolation and screening of naturally occurring antifungal actinomycetes
- Taxonomy of the bioactive actinomycetes
- Production, optimization, extraction and purification of the antibiotic
- Characterization of the antibiotic
Soil samples were collected from different locations in three Indian states viz; Maharashtra (Sinhgad, Pavana river, Mutha river, Mula river and Pune university campus), Karnataka (Ulsoor lake, Lal baug and Cubbon park) and Kerala (Bharat river and Nilambur). Soil samples from these locations were collected in sterile plastic bags. These samples were transferred to the laboratory and stored at 4°C until analysis. Maximum actinomycete isolates were obtained from Pune (51.28%), followed by Bangalore (31.41%) and Malapuram (17.31%). Starch casein agar medium showed more number of actinomycete isolates, thus indicating it to be a good medium for isolation of actinomycetes. In all, 312 actinomycetes were isolated and purified by streak plate method. All these isolates were screened for their antifungal activity by using agar disk method. Out of these, 22% (68 isolates) showed antifungal activity. The Pune isolates showed maximum antifungal activity i.e. 12%, followed by the Bangalore (6%) and Malapuram (4%) isolates. Out of the 22% isolates that showed antifungal activity, 16% showed strong activity against molds, whereas 6% showed activity against yeasts, thus indicating that molds are more susceptible to the actinomycetes as compared to yeasts. The antagonistic activity of the actinomycetes was checked by growing them on different media such as starch casein agar, glucose asparagine agar, glycerol asparagine agar, potato dextrose agar, Sabouraud dextrose agar and yeast extract-malt extract agar. The antifungal activity was maximum when grown on starch casein medium.

The target organisms included species of fungi viz; Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Fusarium oxysporum, Candida albicans, Cryptococcus humicolus, Cryptococcus neoformans, Cryptococcus sp., Penicillium sp., Epidermophyton floccosum, Trichophyton mentagrophytes, Trichophyton rubrum and Microsporum gypseum.

All the actinomycete isolates showed growth at a temperature range from 22°C to 40°C. Different morphological, cultural and physiological characteristics were studied as per ISP procedures. Morphological characteristics were observed by using...
slide culture techniques. Growth of aerial mycelium and substrate mycelium along with pigmentation was observed on different media. Out of the 68 isolates with antifungal activity, 20 strains were selected for further studies on the basis of their strong antifungal activity i.e. isolates showing inhibition zone diameter more than 10 mm. Biochemical tests were performed as per ISP procedures. Carbon utilization and nitrogen utilization were performed by using basal medium with filter sterilized carbon and nitrogen sources respectively. Starch hydrolysis, proteolytic activity, melanin pigmentation, nitrate reduction, milk peptonization, cellulose decomposition and gelatin liquefaction tests were performed as per standard procedures. The antibiotic sensitivity test was performed on all the 20 isolates using various standard antibiotics (Hi-Media, Mumbai). Cell wall analysis for identification of sugar and amino acids was carried out using ascending paper chromatography. Based on their growth, slide culture, biochemical, physiological and chemotaxonomic characteristics it was concluded that sixteen isolates belonged to the genus *Streptomyces* and one each from the genus *Streptoverticillium, Nocardia, Micromonospora* and *Catellatospora*. Probabilistic identification matrices were used for identification of *Streptomyces* species. Out of the selected *Streptomyces* isolates, PU (32, 37, 43, 54) and AK 39 showed close match to *Streptomyces rochei* on the basis of their more or less matching with already studied ones. The isolates PU (11, 23, 25) showed close match to *Streptomyces albidojavus*, PU (15, 28) showed close match to *Streptomyces aureofaciens*. The isolates PU 20, AK 18, AK 24 showed close match to *Streptomyces anulatus* and the isolates AK 16, MS 14, MS 17 showed close match to *Streptomyces griseoflavus* (with reference to the Bergey’s Manual of Systematic Bacteriology, 1989).

Six cultures showing different antifungal characters were selected for further studies. *Streptomyces* sp. PU 23 was active against both yeasts and molds, whereas *Streptomyces* sp. AK 39 was active against only dermatophytes. The other four cultures were active against either molds or yeasts. *Streptomyces* sp. PU 23 was selected for production optimization studies. Effect of different parameters such as temperature, pH, agitation, volume of medium, inoculum size, type of inoculum and concentrations of salt, starch, casein and glycerol on antibiotic production was observed by inoculating
the culture in starch casein broth. The culture showed good antibiotic production at temperature 37°C and pH 7. *Streptomyces* sp. PU 23 required an agitation of 250 rpm and glycerol concentration 1.5% for maximum antibiotic production. Antibiotic production started on the 4th day and maximum production was observed after 8 days of fermentation. The experiment carried out to determine if the antibiotic production is extracellular or intracellular indicated that the supernatant of the culture exhibited activity whereas the ethanol extract of the biomass did not exhibit any activity, thus indicating extracellular nature of the antibiotic. Different solvents such as n-butanol, n-hexane, ethyl acetate, petroleum ether, chloroform, benzene and xylene were used to determine the ideal solvent for extraction of the antibiotic from this culture. Maximum antibiotic was extracted using n-hexane in the case of *Streptomyces* sp. PU 23. A dark brown gummy substance was obtained after concentration of the solvent. This gummy substance was dissolved in ethanol and again concentrated, following which the crude antibiotic powder was obtained. Ethyl acetate was found to be the ideal solvent for extraction in case of *Streptomyces* sp. AK 39 and the other four cultures.

The crude antibiotic obtained from *Streptomyces* sp. PU 23 was selected for further studies. The crude antibiotic obtained was checked for purity by thin layer chromatography using ethanol: water: chloroform (40:40:20) solvent system. Purification of the antibiotic was carried out by column chromatography technique using column chromatography grade silica gel. The crude antibiotic was loaded at the top of the column and eluted with ethanol: water (50:50). The fractions collected were checked for their antifungal activity. The active fraction had an Rf value 0.78. The pure powder obtained was stored at 4°C.

Yield of the antibiotic was found to be 1.66 mg/litre. The MIC and minimum fungicidal concentration (MFC) values of the antibiotic against yeasts and molds were determined. *Aspergillus* species were most sensitive to the antibiotic followed by other molds and yeasts. The antibiotic was active at very low concentration such as 20 μg/ml. Effect of temperature and pH on the shelf life of the antibiotic was studied. The antibiotic was fairly stable for a period of twelve months at 4°C. The thermal stability of the antibiotic was between 30°C to 80°C, however the antibiotic lost its antifungal
activity completely after autoclaving at 121°C for 15 minutes. The antibiotic was stable at pH range 5.7 – 8.0 after it was incubated for one hour in phosphate buffer at different pH. The antifungal effect of the antibiotic from *Streptomyces* sp. PU 23 and hamycin is enhanced when the two drugs were used in combination, thus indicating the synergistic effect. The mode of action of the antibiotic is probably by binding to the ergosterol present in the fungal cell membrane resulting in the leakage of intracellular material and eventually death of the cell. There was no significant loss of the antifungal activity of the antibiotic after treatment with detergents and enzymes. The melting point of the antibiotic was found to be 218-220°C. The elemental analysis showed 37.09% carbon and 6.42% hydrogen. The UV (ultra violet) spectral data exhibited strong absorption at $\lambda_{max}$ 211, 216 and 218nm, suggesting a carbon-carbon double bond. The FTIR (Fourier Transform 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. $^1$H NMR (Nuclear magnetic resonance, 500 MHz) spectra of the antibiotic in D$_2$O 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 $^{13}$C NMR (500 MHz) and DEPT (Distortionless Enhancement by Polarization Transfer, 500 MHz) spectra shows only one –CH$_2$ group at 63 ppm and two olefinic carbons at 102.241 and 102.354 ppm. The LC-Mass spectrum shows a molecular ion at m/z 528 indicating that the molecular weight is probably 528. These spectra differs from the known antifungal antibiotics i.e., hamycin, nystatin, Amphotericin B, cycloheximide, nikkomycin, griseofulvin etc.

With the above spectral data, the compound probably has a molecular formula C$_{16}$H$_{32}$O$_{19}$ having one –CH$_2$OH (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 non-proteinic, straight chain polyhydroxy polyether compound with a single double bond, indicating a non-polyene antifungal antibiotic. The antibiotic seems to be novel, as it does not fit into any of the known classes of antifungal antibiotics.