CHAPTER 4

PRODUCTION, OPTIMIZATION,
EXTRACTION AND
PURIFICATION OF THE
ANTIBIOTIC
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PURIFICATION OF THE ANTIBIOTIC

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4.1 Introduction:

Microorganisms are known to produce secondary metabolites, which are being utilized by man since early 1940s. Actinomycetes have been recognized as the potential producers of such metabolites as antibiotics, growth promoting substances for plants and animals, immunomodifiers, enzyme inhibitors and many other compounds of use to man.

Actinomycetes are well established as producers of antimicrobial agents, and are known to produce two thirds of today’s antibiotics. These antibiotics include agents against, bacteria, fungi, viruses, antitumour agent and antimalarial agents. Production of antibiotics has been the great bioactivity of actinomycetes. Today also they are being screened on large scale for finding new antimicrobial agents. Eventhough the rate of discovery of new antibiotics has been decreased, new approaches in screening for antimicrobials, logistic approaches in exploration of new ecological niches and varied microbial sources are showing good results. It is being said that only 10% of the antibiotics have been discovered till now, leaving large percent of antibiotics undiscovered (Franco and Countinho, 1991).

The study of antibiotics and other fermentation products has shown that a seemingly unlimited number of compounds with diverse structures are produced by microorganisms. The continued high rate of discovery of new chemical entities is due to creative screening procedures. These screening procedures incorporate such features as the emphasis on unusual microorganisms, their special propagation and fermentation requirements, supersensitive and highly selective assays and genetic engineering for the biosynthesis of new compounds. Development in early in vivo evaluation, improved isolation techniques, modern procedures for structural determination, computer assisted identification and an efficient multidisciplinary approach has increased the rate of discovery of new antimicrobial compounds (Franco and Countinho, 1991).

Secondary metabolites are being successfully screened for use, in the fields of agriculture as pesticides and herbicides, antiparasitic compounds and in non-infectious human diseases as enzyme inhibitors, immunomodifiers and antihypertensives (Czoch and Mordarski, 1988).
Source organisms

Screening laboratories are intensifying their search for novel microorganisms as it is clear that the source microorganisms and their cultivation is a major ingredient of an effective holistic screening strategy. In the past, large number of microorganisms, mainly actinomycetes and fungi, have been isolated and manipulated for valuable products using methods that are comparatively easy. Continuation of such an approach without excluding previously studied organisms at an early stage would reduce the efficiency of the screen. Alternatively genetic manipulation of easily isolated microorganisms, or fermenting them in a novel way can provide novel antibiotics. A promising approach, however has been to introduce isolation methods that will provide rare genera or species in large number to allow volume screening with the conviction that novel organisms produce novel secondary metabolites.

It is hoped that novel biochemical activities will be discovered from new or unusual microorganisms isolated on the basis of unique ecological conditions in the environment (Goodfellow and Hynes, 1984). Aquatic microorganisms have elaborated metabolites not usually produced by terrestrial organisms. Indigenous microorganisms isolated from water were found to produce novel polysaccharides, antitumour, antimalarial and aminoglycoside antibiotics. Freshwater sediments and freshwaters are the areas for screening of microorganisms and their metabolites (Okami, 1986).

Antibiotic screening

Factors affecting the screening programme are,

i. Screens should include new detection methods and new source organisms in order to detect novel antimicrobial compounds.

ii. It should have the ability to detect desirable and undesirable compounds as early as possible, which would directly increase the efficiency of the screen.

iii. It should be sensitive, selective and specific and tough enough to withstand the interference from other broth constituents.
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It has been well established that even in exhaustively studied broths there exists as yet discovered secondary metabolites as well as the potential to produce several additional compounds. Detection of these compounds depends on our ability to evolve suitable detection assays. The concept of detection system has progressed considerably since the in vitro assays of broths on agar plates, encompasses a wide range of biochemical tests, enzyme inhibitor models, receptor based models, in vivo screens and creative combination of several individual assays.

The explosive growth of genetic engineering, immunology, molecular pharmacology and virology has led to a deeper understanding of the factors governing the interaction of the drug and target site at the molecular level and provide the screening technologists with plenty of mechanistic target sites. This has led to screens to detect molecules that act on these receptor targets.

The application of miniaturized receptor-based technology has been achieved by overcoming the problems of interference due to microbial enzymes, particulate matters, pH extremes and chelating agents and through the parallel development of process and diagnostic technologies.

**Reporter Gene Assay:**

Molecular techniques now permit cloning of receptor, over expression of key enzymes and the construction of reporter gene assays. Reporter gene assays have been used recently to screen for cell wall active antimycotic agents using *S.cerevisiae*, expressing a cloned β-galactosidase (Zaworski and Gill, 1990). By using a reporter gene construct, with viral Transactivating factors, it is possible to search for compounds interfering with viral replication (Berger *et al.*, 1988).

**Supersensitive strains:** Test strains made selectively supersensitive to a particular class of antibiotic by genetic manipulation and selection, yet retaining natural resistance, allows detection of minute amounts of particular compound, an important criterion in antibiotic screening.

A mutant *E.coli*, highly sensitive to a wide range of β-lactam, was instrumental in the discovery of the first monocyclic β-lactam antibiotic. Supersensitive mutant Ps C55
derived from *Pseudomonas aeruginosa*, was also used to screen β-lactam antibiotics (Shejul, 1998).

**Target sites:** The target site for penicillin is penicillin binding protein (PBP), the enzyme DD-carboxypeptidase. Schindler *et al.* (1986) designed an assay method in which a fluorogenic substrate, ANLA i.e. \(\alpha\)-acetyl-\(\epsilon\)-n-4 (7- nitrobenzofuranyl)-L-lysyl-D-ala-D-ala is used. This substrate is hydrolyzed by DD-carboxypeptidase R39 (from *Actinomadura*) in a cell free system to a fluorescent product. The product is detected under UV light with TLC.

**ELISA:** ELISA method has been developed to detect 12, 14 and 16 membered macrolides possessing amino substituted moieties.

**Antifungal screens:** Selective action against fungi alone is difficult to achieve considering the many physiological features common both to these eukaryotic microorganisms and to the infected host. Therefore, target directed screening has focused on certain loci that are specific to the fungal cell. Screens to detect inhibitors of chitin and glucan synthesis have been developed. Selitrennikoff (1983) described a novel method in which cell wall acting antibiotics are detected by their ability to block the regeneration of protoplasts of a temperature sensitive mutant of the OS-1 variant of *Neurospora crassa*. Chitin synthesis inhibitor screens are also used widely.

Examination of plates for morphological abnormalities could differentiate between the response to different antibiotics. Many known antifungal antibiotics induce abnormalities in fungi and yeasts. Morphological changes such as, curling of hyphae, inhibition of branching and regular and irregular swellings are some of the criteria used for screening of antifungal antibiotics.

**Theory of antibiotic production**

Gaden (1959) defined three basic types of processes of microbial products,

i. The first, results in the formation of growth associated products. These are compounds involved in the biosynthesis of daughter cells or the products of energy metabolism associated with undifferentiated growth.

ii. The second, involves product formation from substances for primary metabolism but via alternate pathways.
iii. The third, where primary metabolism and product formation takes place at completely separate times and products are often derived from amphibolic pathways rather than from catabolism.

The production of antibiotics commences at the end of late exponential phase or stationary phase. However, the usual onset of the production phase varies in different organisms, from later part of the growth phase to many hours after rapid growth has ceased. This is because antibiotic formation is delayed, since enzymes specifically involved in antibiotic biosynthesis are repressed or inhibited during growth.

Antibiotic production is regulated by various ways such as, antibiotics exert feedback regulation on their own formation. Catabolic inhibition and repression of enzymes by a rapidly used carbon source like glucose affects the secondary metabolism (Demain et al., 1979). In fact antibiotic biosynthesis might be regulated as a series of inborn errors of metabolism of the organism (Hokenhull, 1963) superimposed on the normal metabolism of the organisms. Since antibiotic can inhibit growth of their producers, it makes biological sense for cells to suppress the formation of these toxic compounds until rapid growth nears completion.

**Resistance developed by producer**

The resistance mechanisms developed by antibiotic producing microorganisms against own antibiotic are not different from those in clinically resistant bacteria or test organism. The various ways of resistance mechanisms are –

i. Permeability modifications are involved in many instances wherein antibiotics are pumped out of cells against a concentration gradient and a decrease in inward permeability during idiophase protects the organisms from high extracellular concentration of its antibiotics.

ii. Synthesis of enzymes that modify the antibiotic into inactive or less active derivatives.

iii. Modification in the machinery of the targets of the antibiotics.

iv. Feedback inhibition and repression.
Genetic aspects of antibiotic formation

In several antibiotic pathways, intermediates that accumulate in blocked mutants diffuse out of the cell and can be used by other mutants blocked earlier in the pathway. Identification of these intermediates allows elucidation of the biosynthetic pathways of antibiotics (Demain, 1983).

Antibiotic fermentation

Solid-state antibiotic formation:

Reports on antibiotic formation in solid medium have been published (Hobbs et al., 1994). It has been reported that some microorganisms produce antibiotics when grown on agar but not in submerged culture (Hobbs et al., 1994).

Submerged culture technique for antibiotic production:

This is the commercial method of antibiotic production in industries. Cultural and nutritional conditions play an important role in the efficiency of antibiotic production by submerged techniques. Optimization experiments are usually carried out in submerged cultures in which different cultural and nutritional conditions are easily monitored. Large scale productions are carried out by this technique because recovery and purification procedures are simplified in this technique.

Also, stationary mat cultures in liquid medium show antibiotic production. However, it has usually shown lower antibiotic production as compared to shake cultures of the antibiotic fermentation medium (Hobbs et al., 1994).

Optimization of antibiotic production

Antibiotic production efficiency of the producing organism is very sensitive to variations in conditions like cultural and nutritional.

Antibiotic production in large quantities needs suitable media and favourable conditions. The construction of the medium depends on the type of a final product or process and the production can be altered qualitatively and quantitatively by the nature of the growth medium.
4.2 Materials and Methods:

4.2.1 Screening of antibiotic producing actinomycetes

All the actinomycetes were screened for antifungal activity by various recommended methods like giant colony method, agar disc diffusion technique and agar well diffusion technique as mentioned under section 2.2.6. Out of the 20 strong bioactive actinomycetes, six cultures were selected for further studies based on their stability and different antifungal characters.

The six actinomycete cultures selected were (a) *Streptomyces* sp. PU23, (b) *Streptomyces* sp. AK39, (c) *Streptoverticillium* sp. AK 27, (d) *Streptomyces* sp. AK 16, (e) *Streptomyces* sp. MS 14, (f) *Streptomyces* sp. MS 17.


4.2.2 Preparation of inoculum.

Inoculum for antibiotic production was used in the form of spores. The optimization studies were carried out for *Streptomyces* sp. PU 23.

*Streptomyces* sp. PU 23 was selected for the optimization studies because of its ability to strongly inhibit both yeasts and molds. The actinomycete isolate was inoculated on starch casein agar plates and incubated at 37°C for 7 days. Spores were harvested in sterile 0.01% (v/v) Tween-20 in distilled water. The suspension was adjusted to 0.2 $A_{450}$ and used at 10% concentration to get $10^6$ spores ml$^{-1}$ of fermentation medium (Shejul, 1998; Imanura *et al.*, 1993).
4.2.3 Assay of antifungal activity of the supernatant from *Streptomyces* sp. PU 23

Spore suspension of *Streptomyces* sp. PU 23 was inoculated in 100 ml starch casein broth (10^6 spores/ml) in 500 ml flask. The flasks were incubated on shaker (250 rpm) for 7 days at 37°C. The supernatant was obtained at the end of fermentation by centrifuging the fermented broth at 10,000 rpm for 20 minutes. Two-fold dilutions of the supernatant were made in sterile distilled water. 100 μl of each dilution was introduced in agar wells (10 mm) in Sabouraud dextrose agar plates (pH 5.6) seeded with spore suspension (10^6 spores/ml) of *Aspergillus niger*. These plates were incubated at 28°C for 3 days. The inhibition zone diameters were measured and calibration curve of inhibition zone diameter against aliquot of supernatant was constructed. The data was subjected to regression analysis in order to determine the slope, which indicates inhibition zone corresponding to unit increase in the aliquot of supernatant. This corresponds to arbitrary units (Augustine *et al.*, 2004; Motta and Brandelli, 2002).

4.2.4 Optimization of antibiotic production

Antibiotic production depends on type of the media used for fermentation. As per our studies the actinomycete isolates when grown on starch casein medium showed maximum antifungal production as compared to other media used viz; glucose asparagine, glycerol asparagine, potato dextrose, Sabourauds dextrose and yeast extract-malt extract (Table 4.2). Antibiotic production was carried out in 100ml starch casein broth in 500ml Erlenmeyer flasks. The inoculated medium was incubated at 37°C on rotary incubator shaker (Steelmet, India) at 250rpm for 7 days in the 500ml Erlenmeyer flasks. Fermentation broth was centrifuged at 10,000 rpm for 20 minutes and supernatant assayed for antifungal production by using agar well diffusion technique (Augustine *et al.*, 2004).

4.2.4.1 Optimization of temperature for antibiotic production

Five 500 ml Erlenmeyer flasks, each with 100 ml of starch casein medium were inoculated with the actinomycete spore inoculum to get 1x10^6 spores ml⁻¹ of the fermentation medium. Flasks were incubated at 24, 28, 32, 37 and 42°C on rotary
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incubator shaker for 7 days. Fermentation broth was centrifuged at 10,000 rpm for 20 minutes and supernatant was analyzed for antibiotic content by using agar well diffusion technique (Yoshida et al., 1972).

4.2.4.2 Optimization of pH for antibiotic production

The pH of the starch casein fermentation medium was adjusted to 4, 5, 6, 7, 8 and 9 separately with 0.1N NaOH and 0.1N HCl. Fermentation medium was inoculated with spore inoculum (1X 10^6 spores ml^-1). Fermentation flasks were incubated at 37°C on rotary incubator shaker (250 rpm) for 7 days. After 7 days, fermentation broth was centrifuged at 10,000 rpm for 20 minutes and supernatant was analyzed for antibiotic content by using agar well diffusion technique (Takahashi et al., 1994).

4.2.4.3 Optimization of volume of medium for antibiotic production

Starch casein fermentation medium 50, 100, 150, 200, 250 and 300 ml was dispensed in 500 ml Erlenmeyer flasks separately. Flasks were sterilized at 121°C for 15 minutes. Spore inoculum was used to inoculate each flask to get 1X 10^6 spores ml^-1 of the medium. Flasks were incubated at 37°C on rotary shaker incubator for 7 days. After 7 days of fermentation, the broth was centrifuged at 10,000 rpm for 20 minutes. The supernatant was analyzed for antibiotic content by using agar well diffusion technique.

4.2.4.4 Optimization of agitation for antibiotic production

Extent of agitation of the fermentation medium is known to have its effect on antibiotic production. Starch casein fermentation medium was sterilized, inoculated and incubated on rotary incubator shaker at 100, 150, 200, 250 and 300 rpm for 7 days. Incubation temperature was 37°C. After 7 days, fermented broth was centrifuged at 10,000 rpm for 20 minutes and supernatant was analyzed for antibiotic content by using agar well diffusion technique (Beman et al., 1994).
4.2.4.5 Optimization of inoculum size for antibiotic production

Effect of inoculum size was studied on antibiotic production. Starch casein fermentation medium was dispensed in 500ml Erlenmeyer flasks. Spore suspension of 0.2 OD at A_600 was inoculated in 2, 4, 6, 8, 10, 12, 14 and 16% (v/v) proportion to the fermentation medium. Flasks were incubated at 37°C (250 rpm) on a rotary incubator shaker for 7 days. After 7 days, fermentation broth was centrifuged at 10,000 rpm for 20 minutes and supernatant was analyzed for antibiotic content by using agar well diffusion technique.

4.2.4.6 Effect of glycerol concentration on antibiotic production

Effect of glycerol concentration on antibiotic production was studied in starch casein fermentation medium. Glycerol in 1.0, 1.1, 1.2, 1.3, 1.4, 1.5 and 1.6% (v/v) was added to the fermentation medium. Medium was sterilized and inoculated with the actinomycete isolate (as mentioned in 4.2.4.1). Fermentation flasks were incubated at 37°C (250 rpm) for 7 days. After 7 days, fermentation medium was centrifuged at 10,000 rpm for 20 minutes and supernatant was analyzed for antibiotic content by agar well diffusion technique.

4.2.4.7 Effect of starch concentration on antibiotic production

To study the effect of starch on antibiotic production, it was added to the fermentation medium at 0.5, 1, 1.5, 2.0 and 2.5% (w/v). Medium was sterilized and inoculated with the actinomycete isolate spore suspension (as mentioned in 4.2.3.1) and incubated at 37°C on a rotary incubator shaker (250 rpm) for 7 days. After 7 days, the fermentation broth was centrifuged at 10,000 rpm for 20 minutes and supernatant was analyzed for antibiotic content by agar well diffusion technique.

4.2.4.8 Effect of casein concentration on antibiotic production

To study the effect of casein on antibiotic production, it was added to the fermentation medium at 0.05, 0.10, 0.15, 0.20 and 0.25% (w/v). Medium was sterilized and inoculated with the actinomycete isolate spore suspension (as mentioned in 4.2.4.1)
and incubated at 37°C on a rotary incubator shaker (250 rpm) for 7 days. After 7 days the fermentation broth was centrifuged at 10,000 rpm for 20 minutes and supernatant was analyzed for antibiotic content by agar well diffusion technique.

4.2.4.9 Effect of the type of inoculum on antibiotic production

Types of inocula have been found to influence antibiotic production. Inoculum of the actinomycete isolate was developed in mycelial form and spore form. Both these forms of inocula were used independently in starch casein fermentation medium (as mentioned in 4.2.4.1). Fermentation flasks were incubated at 37°C on a rotary incubator shaker (250 rpm) for 7 days. After 7 days, fermented broth was centrifuged at 10,000 rpm for 20 minutes and supernatant was analyzed for antibiotic content by agar well diffusion technique.

4.2.4.10 Effect of salt concentration on antibiotic production

Effect of salt concentration was studied at different concentrations of NaCl viz., 0, 1, 2, 3, 4 and 5% (w/v) in starch casein fermentation broth. Following inoculation (as mentioned in 4.2.4.1), the fermentation flasks were incubated at 37°C on a rotary incubator shaker (250 rpm) for 7 days. After 7 days fermented broth was centrifuged at 10,000 rpm for 20 minutes and supernatant was analyzed for antibiotic content as mentioned above (Fernandez Chimeno et al., 2000).

4.2.5 Determination of MIC values of the supernatant from Streptomyces sp. PU 23 against fungi

The MIC values of the supernatant were determined by broth tube dilution procedure using two fold dilution in Sabouraud dextrose broth at 28°C and compared with that of nystatin.

4.2.6 Time course of antibiotic production

The 500 ml Erlenmeyer flask with 100 ml starch casein broth was inoculated with spores at the rate of 1x10⁶ spores ml⁻¹ of production medium. The flasks were
incubated at 37°C on shaker at 250 rpm. After every 24 hours, 100 μl of the culture broth was withdrawn and analyzed for antibiotic content by agar well diffusion method and biomass in terms of OD₅₄₀ for 12 days. Besides, the pH of the broth was monitored by digital pH meter.

4.2.7 Testing for extra and intra cellular antibiotic production

To test if the antibiotic production was intracellular or extracellular, 100 ml of starch casein fermentation broth in 500 ml Erlenmeyer flask was inoculated with spore inoculum to get 1x10⁶ spores ml⁻¹ of the fermentation medium. The flask was then incubated at 37°C for 7 days at 250 rpm on a rotary incubator shaker. After incubation the broth was centrifuged at 10,000 rpm for 20 minutes and the supernatant as well as the ethanol extract of the biomass were checked for antifungal activity by agar well diffusion method (Omura, 1986). The fungal plates were incubated at 28° C for 24 hours in case of yeasts and 96 hours in case of molds.

4.2.8 Extraction of the antibiotic from culture supernatant using different solvents

Maximum antibiotic production was observed on the 8th day of incubation. Fermentation was terminated on the 8th day and the broth was centrifuged at 10,000 rpm for 20 minutes to separate the mycelial biomass. Different solvents were used and tested for the extraction of the antibiotic from the culture supernatant. The solvents used were n-butanol, n-hexane, ethyl acetate, petroleum ether, chloroform, benzene and xylene to determine the ideal solvent for extraction of the antibiotic from the culture supernatant (Table 4.3). The solvent was added to the supernatant in 1:1 proportion. Solvent-supernatant mixture was agitated for 45 minutes with homogenizer. The solvent was separated from broth by separating funnel. Solvent was centrifuged at 5000 rpm for 15 minutes to remove traces of fermentation broth. All extracts were assayed for their antifungal activity using respective solvents as control by agar well diffusion method as described under 2.2.6.3.
4.2.9 Separation of antibiotic from solvent

The solvent, n-hexane was evaporated by subjecting the sample to rotating flash evaporator (Buchi, Switzerland) at 40°C (50 rpm) under vacuum. The dark brown gummy substance obtained was dissolved in ethanol and concentrated, following which the crude antibiotic powder was obtained. The crude antibiotic was collected and dried in vacuum oven at 40°C overnight. The residue obtained (crude antibiotic) was subjected to purification.

4.2.10 Purification of antibiotic

The crude antibiotic was tested for number of components present by using precoated thin layer chromatography plates (Polygram® Sil G/UV 254, Macherey-Nagel) using ethanol: water: chloroform (40:40:20) solvent system. Purification of the antibiotic was carried out by column chromatography using silica gel of column chromatography grade (SRL, Mumbai). Column (35 x 10 mm) was cleaned using water and rinsed with acetone. After drying, a small piece of cotton was placed at the bottom of the column. Silica gel was then packed in the column by using ethanol: water (50:50) as solvent system. The crude antibiotic was loaded at the top of the column and eluted using ethanol: water (50:50) as solvent system. Fractions were collected at 20 minutes interval. Thin layer chromatography (TLC) of each fraction was performed using precoated TLC plates and simple glass plates to detect the antibiotic. The TLC plates were exposed to iodine vapors to develop the antibiotic, if any. The fractions having same Rf value were mixed together and the solvent evaporated at 40°C in a vacuum oven. These fractions were tested for their antifungal activity by using the agar well diffusion method. The fractions showing antifungal activity were again purified by using the same above mentioned column chromatography system and purity was confirmed using TLC plates. The brown coloured powder obtained was stored in an ampoule at 4°C.
4.3 Results

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 (Table 4.1). The supernatants of these actinomycete cultures inhibited mycelial growth and budding in case of molds and yeasts, respectively. This was observed under the phase contrast microscope (Nikon, Japan).

4.3.1 Response curve of Aspergillus niger against antifungal activity of Streptomyces sp. PU 23

For the purpose of reliable bioassay, it was necessary to construct response curve for the antibiotic. This was done by diluting the supernatant of the fermented broth (as mentioned in 4.2.3) and testing these dilutions against Aspergillus niger. A dose-response relationship between the inhibition zone diameter and the concentration of supernatant was linear in the dilution range used, based on which one unit of antifungal activity was defined as the amount corresponding to 0.3 mm (diameter) of inhibition zone of Aspergillus niger under the assay conditions (Fig 4.1).

4.3.2 Optimization of antibiotic production

Streptomyces sp. PU 23 exhibited good antifungal activity when grown on starch casein medium against yeasts and molds (Table 4.2). The effect of various parameters on the production of the antibiotic from Streptomyces sp. PU 23 was as follows:

4.3.2.1 Effect of temperature on antibiotic production

The fermentation was carried out at temperatures ranging from 24 to 42°C. The actinomycete culture showed maximum antibiotic production (600 units/ml) at 37°C, indicating it to be the optimum temperature (Fig 4.2).
4.3.2.2 Effect of pH on antibiotic production

Fermentation media with varied initial pH (4 to 9) were used for antibiotic production. The culture supernatant of the actinomycete culture was analyzed for antibiotic content. The broth from medium of pH 7 showed maximum antibiotic production (600 units/ml). The production was relatively less at pH above and below 7 (Fig 4.3).

4.3.2.3 Effect of volume of medium on antibiotic production

The antibiotic fermentation was carried out in 500 ml Erlenmeyer flasks with varied air space to fermentation medium volume ratios. Maximum antibiotic production (600 units/ml) was observed with 1:1 ratio of air space and volume of the medium, i.e. 250 ml fermentation medium in 500 ml Erlenmeyer flask (Fig 4.4).

4.3.2.4 Effect of agitation on antibiotic production

*Streptomyces* sp. PU 23 required an agitation of 250 rpm for maximum antibiotic production (Fig 4.5).

4.3.2.5 Effect of inoculum size on antibiotic production

Varied volumes of spore inoculum (0.2 OD at A540) were used for antibiotic production. Maximum antibiotic production (600 units/ml) was observed with 10% inoculum size, which remained same with further increase in inoculum size (Fig 4.6).

4.3.2.6 Effect of glycerol concentration on antibiotic production

The fermentation was carried out using varied concentrations of glycerol (1.0 to 1.6% v/v). Maximum antibiotic production (600 units/ml) was observed with 1.5% glycerol concentration (Fig 4.7).
4.3.2.7 Effect of starch concentration on antibiotic production
The fermentation was carried out using varied concentrations of starch (0.5 to 2.5% w/v). Maximum antibiotic production (600 units/ml) was observed with 1% starch in the fermentation medium (Fig 4.8).

4.3.2.8 Effect of casein concentration on antibiotic production
The fermentation was carried out using varied concentrations of casein (0.05 to 0.25% w/v). Maximum antibiotic production (600 units/ml) was observed with 0.1% casein in the fermentation media (Fig 4.9).

4.3.2.9 Effect of the type of inoculum on antibiotic production
Fermentation was carried out using inoculum in the form of spores and filamentous cells. There was no significant difference in the antibiotic production of both the forms of the culture (Table 4.4).

4.3.2.10 Effect of salt concentration on antibiotic production
The fermentation was carried out using varied concentrations of NaCl (0 to 5% w/v). As the concentration of NaCl increased, the antibiotic production decreased. Thus, maximum antibiotic production (600 units/ml) was observed at 0% NaCl (Table 4.5).

4.3.3 MIC values of the supernatant from \textit{Streptomyces} sp. PU 23 against fungi
The MIC values of the supernatant were less as compared to nystatin in the case of molds and more in the case of yeasts (Table 4.6).

4.3.4 Time course of antibiotic production
The antibiotic production was monitored over a period of 12 days. Maximum antibiotic was produced on the 8th day. The rate of antibiotic production correlated with growth rate of \textit{Streptomyces} sp. PU 23 and was highest (600 units/ml) in the late log phase. The pH of the broth was between 6.8 and 7 throughout the fermentation (Fig 4.10).
4.3.5 Testing for extra and intra cellular antibiotic production

The experiment carried out for checking extra and intra cellular antibiotic production showed that the supernatant of the culture inhibited all the pathogenic fungi tested while the ethanol extract of the biomass showed no antifungal activity. This result showed that the antibiotic production of the actinomycete was extra cellular in nature.

4.3.6 Extraction of the antibiotic

Different solvents were used and tested for the extraction of antibiotics. The solvents used were n-butanol, n-hexane, ethyl acetate, petroleum ether, chloroform, benzene and xylene to determine the ideal solvent for extraction of the antibiotic from the culture supernatant. The solvents containing the extracted antibiotic were tested against the fungal test cultures using the respective solvent as control. In case of *Streptomyces* sp. PU 23, maximum antibiotic yield was observed in residue, which was extracted by using n-hexane (Table 4.3).

4.3.7 Purification of the antibiotic

The crude antibiotic obtained from *Streptomyces* sp. PU 23 was used for further studies. The fractions collected by column chromatography technique were checked for their antifungal activity. The active fraction had a $R_f$ value 0.78. The pure powder thus obtained was stored at 4°C.
Plate 4.1 Antifungal activity exhibited by n-hexane extract of supernatant from Streptomyces sp. PU23 against (a) Aspergillus niger and (b) Candida albicans.

Plate 4.2 Crude antibiotic powder from Streptomyces sp. PU23.
### Table 4.1 Properties of antifungals from actinomycete isolates

<table>
<thead>
<tr>
<th>Actinomycete isolates</th>
<th>Active against</th>
<th>Solubility in</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces</em> sp. PU 23</td>
<td>Yeasts and molds</td>
<td>n-Hexane</td>
</tr>
<tr>
<td><em>Streptomyces</em> sp. AK 39</td>
<td>Dermatophytes</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td><em>Streptomyces</em> sp. AK 27</td>
<td>Molds</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td><em>Streptomyces</em> sp. AK 16</td>
<td>Yeasts</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td><em>Streptomyces</em> sp. MS 14</td>
<td>Molds</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td><em>Streptomyces</em> sp. MS 17</td>
<td>Yeasts</td>
<td>Ethyl acetate</td>
</tr>
</tbody>
</table>

### Table 4.2 Antibiotic production by *Streptomyces* sp. PU 23 grown in different media for 7 days

<table>
<thead>
<tr>
<th>Growth medium for <em>Streptomyces</em> sp. PU 23</th>
<th>Yield of antibiotic (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch casein</td>
<td>600</td>
</tr>
<tr>
<td>Yeast extract-malt extract</td>
<td>333</td>
</tr>
<tr>
<td>Sabouraud dextrose</td>
<td>233</td>
</tr>
<tr>
<td>Glycerol asparagine</td>
<td>300</td>
</tr>
<tr>
<td>Glucose asparagine</td>
<td>333</td>
</tr>
<tr>
<td>Potato dextrose</td>
<td>266</td>
</tr>
</tbody>
</table>

The culture supernatant obtained by centrifugation of the broth culture at 10,000 rpm for 20 minutes.
Table 4.3 Different solvents used for extraction of antibiotic from *Streptomyces* sp. PU 23

<table>
<thead>
<tr>
<th>Solvents used for extraction of antibiotic</th>
<th>Yield of the antibiotic (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-hexane</td>
<td>600</td>
</tr>
<tr>
<td>Petroleum ether</td>
<td>200</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>-</td>
</tr>
<tr>
<td>n-butanol</td>
<td>-</td>
</tr>
<tr>
<td>Chloroform</td>
<td>-</td>
</tr>
<tr>
<td>Benzene</td>
<td>-</td>
</tr>
<tr>
<td>Xylene</td>
<td>-</td>
</tr>
</tbody>
</table>

The solvents chloroform, benzene and xylene were themselves inhibitory to the target organisms and hence considered poor solvents for extraction.

Fig 4.1 Response curve of *Aspergillus niger* against antifungal activity of *Streptomyces* sp. PU 23
Production, optimization.....

Fig. 4.2 Effect of temperature on antibiotic production by *Streptomyces* sp. PU 23

![Diagram of antibiotic production vs. temperature](image)

Fig. 4.3 Effect of pH on antibiotic production by *Streptomyces* sp. PU 23

![Diagram of antibiotic production vs. pH](image)

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**Fig 4.4** Effect of ratio of space to volume of medium on antibiotic production by *Streptomyces* sp. PU 23

![Bar chart showing antibiotic production vs. volume of medium.](chart1)

**Fig 4.5** Effect of agitation on antibiotic production by *Streptomyces* sp. PU 23

![Bar chart showing antibiotic production vs. agitation rate.](chart2)
Production, optimization...

Fig 4.6 Effect of inoculum size on antibiotic production by *Streptomyces* sp. PU 23

Fig 4.7 Effect of glycerol concentration on antibiotic production by *Streptomyces* sp. PU 23
Fig 4.8 Effect of starch concentration on antibiotic production by *Streptomyces* sp. PU 23

Fig 4.9 Effect of casein concentration on antibiotic production by *Streptomyces* sp. PU 23
Table 4.4 Effect of type of inoculum on antibiotic production by *Streptomyces* sp. PU 23

<table>
<thead>
<tr>
<th>Type of inoculum</th>
<th>Antibiotic production (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spore form</td>
<td>600</td>
</tr>
<tr>
<td>Mycelial form</td>
<td>600</td>
</tr>
</tbody>
</table>

Table 4.5 Effect of salt concentration on antibiotic production by *Streptomyces* sp. PU 23

<table>
<thead>
<tr>
<th>NaCl concentration (%w/v)</th>
<th>Antibiotic production (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>600</td>
</tr>
<tr>
<td>1</td>
<td>433</td>
</tr>
<tr>
<td>2</td>
<td>300</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig 4.10 Time course of antibiotic production by *Streptomyces* sp. PU23
Table 4.6 MIC values* of the supernatant** from *Streptomyces* sp. PU 23 against fungi in comparison with that of nystatin.

<table>
<thead>
<tr>
<th>Target organisms</th>
<th>MIC (units/ml)</th>
</tr>
</thead>
<tbody>
<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 humicolus</em> NRRL 12944</td>
<td>3.75</td>
</tr>
</tbody>
</table>

* Determined by broth tube dilution procedure using two-fold dilution in Sabouraud dextrose broth at 28°C.

**Inoculum (1x10⁶ 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.
4.4 Discussion

The actinomycetes were isolated on starch casein agar. Most of the isolates, showed good bioactivity. The antifungal metabolite was active in the agar medium and the fermentation broth unlike fumaradimycin, which is inactivated in the fermentation broth (Maruyama et al., 1975). The actinomycete isolate showed antifungal activity but was not antibacterial. The production of the antibiotic was influenced by media components and cultural conditions, such as aeration, agitation, pH, temperature and glycerol concentration. Such conditions are known to affect antibiotic production and these fermentation conditions vary from organism to organism (Iwai and Omura, 1982).

Cultural conditions such as pH, temperature, aeration, type of inoculum and agitation was found to affect the antibiotic production by this isolate. The optimum pH for antibiotic production was found to be 7. It is well known that the pH affect antibiotic production in all cases. It has been reported that the change in pH of the culture medium induces production of new substances that adversely affect antibiotic production (Omura et al., 1973). Change in pH affects the production of secondary metabolites in the cells. It has been reported that the production of helvoic acid and cerulenin by Cephalosporium caerulescens was affected by change in the pH (Iwai et al., 1973). Production of helvoic acid was increased whereas cerulenin was little affected.

In Streptomycetes sp. PU 23, the optimum temperature for antibiotic production and growth was found to be same, i.e. 37°C. Slight deviation in temperature from the optimum temperature for growth does not affect growth to that extent but this change severely affects antibiotic production and thereby the yield of the antibiotic (Yoshida et al., 1972). Agitation affects aeration and mixing of the nutrients in fermentation medium.

The optimum rate of agitation (250 rpm) was found to facilitate increased antibiotic production in the 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 was increased with the increased dissolved oxygen (Stevens et al., 1972).
Starch casein fermentation medium was widely used for the production of antibiotic by the present actinomycete isolate. When this media was supplemented with various concentrations of casein (0.05 to 0.25%) and starch (0.5 to 2.5%) the activity of the antibiotic was variably affected. After optimizing the type and the size of the inoculum it was observed that the activity was maximum when 0.1% of casein and 1% starch was used in the medium. The MIC (minimum inhibitory concentration) values of the supernatant was less as compared to nystatin in the case of molds and more in the case of yeasts (Table 4.6).

Rate of production of the antibiotic was directly proportional to the growth rate. Maximum antibiotic production (600 units/ml) was achieved at the late log phase, which remained constant during stationery phase (Fig 4.10). However, there are many reports about the antibiotic production in the stationery phase as antibiotic is a secondary metabolite (Reichenbach et al., 1988). The antifungal activity of the strain was extra cellular in nature. In most of the cases, antibiotics are extra cellular (Hacene et al., 2000; Gupta and Naik, 1999; Paradkar et al., 1998; Kearns, 1977).

The antibiotic from *Streptomyces* sp. PU 23 was extracted from the supernatant using n-hexane, a non polar solvent and not extracted using ethyl acetate. However, most of the antifungal antibiotics are extracted using ethyl acetate (Franco and Coutinho, 1991). The pure compound is soluble in water but the standard antifungal antibiotics, like hamycin, are insoluble in water. The peaks of the ultraviolet spectrum of the compound differs from the peaks of the standard antifungal antibiotics like nystatin, amphotericin B and hamycin, thus indicating it to be a non-polyene antifungal antibiotic.

The purified antibiotic was active against a number of test organisms like *Aspergillus niger*, *Aspergillus fumigatus*, *Fusarium oxysporum*, *Candida albicans* and *Cryptococcus* species.
Production, optimization.....

4.5 References:
Production, optimization…..


