CHAPTER 5

CHROMATOGRAPHIC AND SPECTROPHOTOMETRIC STUDIES OF ANTIFUNGAL ANTIBIOTIC SUBSTANCES
INTRODUCTION

The antibiotics can be defined broadly as substances produced by one organism that have adverse effect on other organisms, (Hopwood, 1989 and Davies, 1990). This operational definitions, however, does not reflect the role of antibiotics in the natural environment which remains poorly understood, although at least some of these substances confer a competitive advantage to the producer organism (Martin and Demain, 1980).

Many of the compounds classified as antibiotics are peptides or small proteins. These often contain amino-acid residues not commonly found in proteins (Kalter and Moreno, 1992).

A number of antibiotics have become important therapeutic agent. Several antibiotics have been found to be clinically effective in protozoan and antibacterial infections. Unlike therapy for bacterial infections, there have been relatively few therapeutic agents developed for the treatment of infections caused by fungi. In recent years fungal infections are world-wide in nature and are systematic as well as local among individuals of all ages. Many remedies have been used against fungal infections and research still continues but ideal topical antifungal agent has not yet been found.
All the polyene producers exhibited strong antifungal nature (Baiyan, 1977). Many polyene antibiotics having a conjugated system of double bonds show antifungal activity. They are macrocyclic lactones but are different from the macrolide antibiotics of the erythromycin type by having a larger lactone ring in which conjugated polyene system is present. Many of them have a glycosidically linked amino sugar. Their antifungal action is perhaps by forming a complex with sterols which are present in the membrane. Three important macrocyclic antibiotics which are used as antifungal agents are nystatin (Mycostatin), amphotericin-B (Fungizone) and candididin (Candeptin). The other important antifungal agent is 5-fluorocytosine which affects RNA synthesis and griseofulvin, which interacts with microtubules and inhibits cell division in different type of cells (Chatwal, 1992).

Polyene antibiotics include about 150 substances. Many of them contain amino sugar (Mycosamine, Perosamine) and sugar substances have the second nitrogen compound (aromatic ketones such as p-aminoacetophenone and p-aminophenylacetone). Polyene antibiotics, depending on the presence of some conjugated double bonds in their structure can be subdivided into six groups (Egorov, 1985) i.e. trienes
(microtriene, trienin, triene etc.), tetraenes
(arenomycin, nystatin, fumagillin etc.), pentaenes
(mycoticin, flavomycin, fungochromin etc.), hexaenes
(dermostatin, endomycin - B, flavicide etc.),
heptaenes (candidin, penimycin, tricomycin etc.) and
octaenes (ochramycin).

Most antifungal antibiotics of the
streptomycetic origin are polyene by their chemical
structure. During studies of the culture of certain
streptomycetes, the existence of antibiotic substances
as by product of metabolism has been observed by mean
of chromatography techniques.

Chromatography plays a very important and
significant role in the field of analysis,
particularly for separation, identification and
purification of chemical compounds. The satisfactorily
and simplicity of process and quick identification of
constituents is now currently employed in the
laboratory. It is used to identify the antibiotic at
an early stage of its synthesis by a microorganism.

The techniques which are followed are mainly
of paper or thin layer chromatography. A reliable
technique for the identification of antibiotics or
their producer strains by chromatography was
discovered in 1903 by Russian scientist Tswett
(Egorov, 1985). This method is so fruitful and common that by this time chromatography has been used in almost every type of compound particularly in medicine and biological sciences.

The chromatographic methods in antibiotic research is used for the separation of accompanying impurities formed during fermentation and purification of an antibiotic compound. Penicillin was earlier purified by repeated chromatography on a column of alumina by Abraham and Chain (1942). Column chromatography was also used for the separation of different penicillins (Fishchbach et al., 1946 - 1947; James et al., 1951).

Titus and Fried (1947) used the paper partition chromatograms for the separation of mannosido - streptomycin from streptomycin. Wisten and Eigen (1948); Horne and Pollard (1948) used above method to study the streptocin complex/types of streptomycin and Kluener (1949) used to investigate different penicillin preparations. Bird and Pugh (1954); Fishbach and Levine (1955); Selzer and Wright (1957); Kelly and Buyske (1960) used paper chromatography for the separation of tetracyclines. Burton and Abraham (1951) succeeded in separating related cephalosporins with the help of paper
chromatography. Vining and Waksman (1954) separated actinomycins by paper chromatography. Vining et al. (1955) studied canditin - candididin group; Blinov et al. (1962) for antibiotics having indicator properties.

Ito et al. (1964) studied the separation of basic water soluble antibiotics with the help of thin layer chromatogram. Inouye and Ogawa (1964) utilized TLC for the amino sugar antibiotics and their degradation products. Kondo et al. (1964) studied the same group of antibiotics by TLC. Semenova et al. (1963) prepared chromatograms of certain actinomycetes producing closely related antibiotics; later on Staneck and Roberts (1974) made an effort to identify the aerobic actinomycetes by thin layer chromatography. Ammann and Gottlieb (1955); Paris and Teallet (1962); Shin - Ichi et al. (1964) have also used thin layer chromatography for the analysis of antibiotic. Mayes and Smith, (1964) and Betina and Barath (1964) described various methods of bioautographic development of thin layer chromatograms.

Physical methods can also be used for the detection of antibiotics on paper and thin layer chromatogram. Physical methods of identification of
antibiotics comprise techniques associated with (a) luminescence in ultraviolet rays (b) absorption of ultraviolet radiation and (c) radioactive tracing of antibiotics. Out of these three ultraviolet radiation absorption technique is commonly used for the detection of antibiotics.

Several workers have classified the polyenic antifungal antibiotics in a number of different groups according to the nature of their ultraviolet absorption spectra. (Utahara et al., 1954; Oroshnik et al., 1955; Vining et al., 1955; Pledger and Lechevalier, 1956; Ball et al., 1957; Waksman and Lechevalier, 1962; Balyan, 1977 and Gupte, 1991). In the present study a similar method was used in screening the antifungal antibiotics produced by various *Streptomyces* strains.

In the present investigation seven promising antagonists, specially antifungal *Streptomyces* strains i.e., Ac1, Ac5, Ac40, Ac43, Ac52, Ac54 and Ac56 were selected for detailed screening. The column, paper and thin layer chromatography techniques were used for the purification and detection of antifungal antibiotic substances. Ultraviolet absorption/spectrophotometric techniques were also used for the primary identification of antifungal antibiotic substances.
A: CHROMATOGRAPHIC SEPARATION AND PURIFICATION OF ANTIFUNGAL ANTIBIOTIC SUBSTANCES

MATERIALS AND METHODS:

To evaluate the antifungal antibiotics the following steps were adopted:

(a) Production of antifungal compounds: In order to study the antifungal antibiotics produced by the Streptomyces strains i.e., Ac1, Ac5, Ac40, Ac43, Ac52, Ac54 and Ac56 the organisms were grown in broth medium. The medium was distributed equally (60 ml) in 250 ml Erlenmeyer flasks aseptically and sterilized at 120 lbs pressure for 20 minutes. The flasks were inoculated with two disks (6 mm) of the culture obtained from the edge of actively growing colonies of Streptomyces strains. All the flasks were kept at 28\(^\circ\) ± 1\(^\circ\)C on a reciprocating shaker at 160 rpm. After 10 days of incubation the culture was harvested, filtrate was separated from the mycelia and spores by filtration through Whatman No.1 filter paper and by centrifugation at 6000 rpm for 30 minutes. The activity of antifungal antibiotic substances present in the culture filtrate was determined by evaluating the inhibition (%) of spore germination of the test fungal pathogens. The crude filtrate was also studied for the extraction of antifungal antibiotics.
(b) Extraction of antibiotics: The crude preparation of antibiotics was obtained from the culture filtrate of 10 days old shaked culture of *Streptomyces* strains. The cultures filtrates was mixed with ethyl acetate (1:1 by volume) in each case in separating funnels, shaken vigorously for 30-45 minutes and kept for over night. The two layers thus formed i.e., supernatant or solvent layer (transparent layer) in the top and aqueous layer at the base of the separating funnel. The solvent layer was separated carefully and taken in a separate flask. The aqueous phase was again poured in separating funnel and the same process was repeated. The solvent layer was separated again carefully and the aqueous phase was rejected as no activity could be recorded in it. The solvent layer was poured in a porcelain dish and evaporated in a vaccume desiccator. The oily residue refused with minimum quantity of benzene was evaporated to dryness in a vaccume desiccator. The condensed material extracted with minimum quantity of absolute alcohol and then concentrated in vaccume dessicator. The condensed material (0.1-0.2 ml) thus obtained, is the crude antibiotic substance. Equal amount of the crude antibiotics thus obtained from the culture filtrate of each strain of the *Streptomyces* was transfered in clean sterilized small screw capped glass tube, one ml
of acetate was added and mixed thoroughly. These ethylacetate extract of antibiotics was further purified.

(c) Purification of antibiotics: Purification of antifungal antibiotics was done using chromatographic columns prepared in a glass tube (45 cm long and 1.5 cm radius) having a nylon grid and a glass stopper at its bottom. The column was clamped in a vertical position and filled with 25 gm of acid washed with alumina in ether, after packing of the column 1 ml condensed extract was poured in the alumina packed column in a step wise manner with the help of a pipette. The stop cock at bottom can be open and the solvent is allowed to run down. The eluting solvent ether-chloroform mixture was then added and allowed to flow through the alumina. This flow will be continue until the mixture is completely separated into its compounds. The fractions were collected in separate flasks and then transferred in the porcelain dishes and evaporated to dryness in a vacuum dessicator. The residue can be dissolved in 0.5 ml ethyl acetate and further used for the chromatographic analysis.

(d) Partial characterization of antibiotics: The active substances can be partially characterized by
paper chromatogram and by thin layer chromatogram (TLC).

(e) Thin layer chromatography: Thin layer chromatography plates (10 x 20 cm) having an uniform coating of 0.3 mm were prepared by using a slurry of silica gel 25 HR (30 gm silica gel in 55 ml distilled water). The plates were dried at room temperature and desiccated. Before use the plates were activated by heating in oven at 100-110°C for about 1/2 hour (Gimeno, 1983; Srivastava and Srivastava, 1987). After cooling of the plates small quantity (50 ul, 100 ul) of antibiotic sample was applied in a series of small spots about 3/4 inch apart along a line and about a inch from one end of a TLC plates, the spot or the chromatogram can be dried in air. The silica gel plates were developed in equilibration tanks, the developing solvent system used was, ethyl acetate : methanol (95 :5 v/v); aceton : water (9 :4 v/v) and n-butanol saturated with water. The chromatographic tank was previously saturated with the desired solvent. After saturation was achieved the TLC plate was placed in the tank having the desired solvent. When the chromatogram developed the plates were removed from the tank and dried at room temperature in horizontal position. The antibiotic spots on a chromatograms were examined and detected by observing them under UV chamber (Widson Scientific works, Delhi).
(f) **R<sub>f</sub> value**: The antibiotic substances from the elute can be spread out and separated along the path of travel of the solvent from the point of spot application or 'origin'. The position of the spot of the test substance is very important to assess the results. The ratio of the distance from the origin that each individual compound in each solvent system, travelled to the total distance and travelled by solvent mixture. The 'solvent front' provides the 'R<sub>f</sub> value'. R<sub>f</sub> coefficient was calculated with the help of following formula:

\[ R_f = \frac{\text{Distance of migration of the solute}}{\text{Distance of migration of the solvent}} \]

(g) **Paper chromatography**: Paper chromatography was used for bioautographic technique for the detection of antibiotic substances. The antibiotic potency was evaluated using bioautographic techniques by cutting the part of a chromatogram having spot and by placing it over the surface of agar medium in the petridishes, previously seeded with the test pathogen i.e., *A. niger*. After incubation at 28°±1°C, the uniform growth of the test organism was observed on the medium.

**RESULTS**

In the present study, the chromatograms were assayed against the test pathogens i.e., *A. niger* and
the active antifungal substances were spotted. The $R_f$ value of the active spots or chromatogram were recorded in Table - 48. Chromatographic details of the test *Streptomyces* strains are as follows:

**Streptomyces** strain Ac1 : The results showed that two spots having blue flourescent developed in solvent system-A. The $R_f$ value of these spots was 0.86 and 0.98. In the solvent system-B three spots were developed. Two spots showed blue flourescent and the other spot showed green flourescent. The $R_f$ value of these spots recorded was 0.92, 0.71 and 0.09. One spot having blue flourescent ($R_f$ value 0.88) also developed in solvent system-C. Distinctive mark of the spot was recorded in all the solvent systems.

**Streptomyces** strain Ac55 : The results showed that a spot ($R_f$ value 0.95) having greenish blue flourescent developed in solvent system-A. In the solvent system-B three spots were noted, all of these were of blue flourescent. The $R_f$ value of these spots was 0.63, 0.77 and 0.86. No spot was seen in solvent system-C.

**Streptomyces** strain Ac40 : The $R_f$ values of 0.74 and 0.95 showing blue flourescent of active spot was noted in solvent system-A. No spot could develop in solvent system-C. In solvent system-B two spots develop ($R_f$ values - 0.11 and 0.90) showing green and blue flourescent. Distinctive mark of spot was also seen.
Table - 48. Chromatographic analysis of the metabolites produced by test *Streptomyces* strains

<table>
<thead>
<tr>
<th><em>Streptomyces</em> strain No.</th>
<th>Solvent system</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>No. of spots</td>
</tr>
<tr>
<td>Ac1 2 0.98 Blue + 0.86 Blue</td>
<td>2 0.98 Blue</td>
</tr>
<tr>
<td>Ac5 1 0.95 Greenish - 0.86 Blue</td>
<td>1 0.96 Blue</td>
</tr>
<tr>
<td>Ac40 2 0.95 Blue + 0.74 Blue</td>
<td>2 0.90 Blue +</td>
</tr>
<tr>
<td>Ac43 1 0.87 Blue + 0.11 Green</td>
<td>1 0.95 Blue</td>
</tr>
<tr>
<td>Ac52 1 0.96 Blue - 0.12 Green</td>
<td>1 0.96 Blue</td>
</tr>
<tr>
<td>Ac54 1 0.80 Blue + Nil -</td>
<td>1 0.80 Blue</td>
</tr>
<tr>
<td>Ac56 1 0.88 Blue + 0.73 Green</td>
<td>1 0.88 Blue</td>
</tr>
</tbody>
</table>

* DMS : A flourescent distinctive mark at the spots left after solvent run.

Solvent system A : ethyl acetate : Methanol (95 : 5 v/v).
B : Acetone : Water (9:4 v/v).
C : n-Butanol saturated with water.
**Streptomyces strain Ac43**: The results showed the appearance of two spots (Rf values 0.06 and 0.93) in solvent system-B, having green and blue flourescent. A distinct spot (Rf value = 0.97) was seen in solvent system-A showing blue flourescent. No spot develop in solvent system-C.

**Streptomyces strain Ac52**: The Rf value 0.96 having blue flourescent and Rf value 0.12 having green flourescent of active substances were observed in solvent system-A and B, respectively. No spot was recorded in solvent system-C.

**Streptomyces strain Ac54**: No spot developed in solvent system-B and C. Solvent system-A was showing a spot (Rf value 0.80) having blue flourescent and a distinctive mark at the spot was also seen.

**Streptomyces strain Ac56**: Rf value 0.88 with blue flourescent of active spot was seen in solvent system-A but none of the spot could be detected in solvent system-C. The two spots (Rf value 0.02 and 0.73) were developed in solvent system-B with green flourescent and the distinctive mark of the spot.

Of the three solvent systems used in (Table - 48) the present study, solvent system-A (Ethyl acetate with methanol, 95 : 5 v/v) was found to be the most effective in separating the antifungal antibiotic substances. Seeing the encouraging results this solvent system was selected for further investigations.
MATERIALS AND METHODS:

Ultraviolet spectrum is one of an important characteristics of the antifungal antibiotics used for the identification of antibiotic substances. This was measured with the help of spectrophotometer. Eight selected antagonists i.e., Ac1, Ac5, Ac40, Ac43, Ac52, Ac54 and Ac56 were selected for the present study.

For studying the ultraviolet (light) absorption, the crude antibiotic substances obtained from the isolates of *Streptomyces* were purified by the column chromatography and then the silica gel 'G' coated plates were spotted with a small amount of purified and concentrated antibiotic substance. The spotted plates were placed vertically in the solvent in a closed chromatographic chamber. The plates were kept in such a way that the spot do not covered by the solvent. The antibiotic containing spots on different plates were detected by irradiation of the plates with ultraviolet light.

The antibiotic spot appearing on the plate was eluted separately and dissolved in equal amount of spectroscopic 95% methanol for UV-light absorption
studies, using Shimadzu Double Beam Spectrophotometer UV-190. The absorption spectrum was determined by measuring the absorbance at different wavelength of light ranging from 250 nm to 340 nm.

RESULTS

Five *Streptomyces* produced tetraene polyene type of antibiotics and two *Streptomyces* produced antibiotic which seem to be of triene polyene in nature.

The UV spectrum showed the maximum absorption at 290, 303 and 320 nm, which is an indicative of tetraene polyene macrolide (Ball et al., 1975; Omura and Tanaka, 1984). Isolates of *Streptomyces* like Ac5, Ac40, Ac43, Ac52 and Ac56 can be of the above nature. The highest wavelength absorption peak at 303 nm was shown by *Streptomyces* Ac5. It indicates the presence of some internal cis-double bond (Gupte, 1991). Some *Streptomyces* strains i.e., Ac5, Ac40, Ac43 and Ac56 showed absorption maxima at 290 nm wavelength, which is an indicative of tetraene polyene type of antibiotics. *Streptomyces* Ac1 and Ac54 showed absorption maxima at 270 nm, it may be a new type of antibiotic. It also differs from other polyene antibiotics and non polyene antifungal griseofulvin antibiotic.
DISCUSSION

Some actinomycetes, specially species of *Streptomyces* have been known to possess a series of highly valuable chemical substances including antibiotics. This property is considered as highly characteristic for a given species. The fact that a large proportion of the cultures of *Streptomyces* isolated from natural substrates showed some degree of inhibition of growth of other microorganisms when tested on suitable media. This suggests the ability to form antibiotics to be of potential diagnostic value (Waksman, 1959).

The prevalence of polyenic antifungal antibiotic producers in soil has been earlier reported by Pledger and Lechevalier, 1955-1956; Rehacck, 1963; Dasgupta *et al.*, 1970. There does not appear to be any correlation of polyene type with geographical location, although the number of soil samples investigated is too small for drawing any firm conclusions. Such relationship is rather improbable in view of the general kind of distribution. Further, the fact that one isolate can produce more than one type of polyene would appear in consistent with any such type of relationship, even though the isolates which produced more than one polyene originated in the Argentine (Ball *et al.*, 1957). These antibiotics can
also be extracted, concentrated and precipitated as in the case of other polyene antibiotics (Thirumalachar et al., 1964). Test for the activity against some strains (particularly Trichophyton mentagrophytes and Candida albicans) of target pathogenic fungi from clinical sources is important for the assessment of the value of the antibiotic (Waksman and Lechevalier, 1962).

The studies of microbial metabolites for Pharmacological activity have been often been the by-products of the programmes studying the compound for therapeutic effects and since the interest has usually involved negative attributes e.g. toxicity. It is not surprising that most of the observations have not been encouraging as far as discovering useful pharmacological agents (Perlman and Peruzotti, 1970). There is an intense interest in obtaining antifungal agents with activity comparable to the polyene antibiotics (Nakayama, 1981).

The first survey of the distribution of antifungal properties among actinomycetes was made by Alexopoulos (1941). A large number of compounds have now been isolated and described under the names of nystatin, candididin, candidin, hamycin, trichomycin and others. Most of them belong to the polyenes. Once
the basis of selective action of an antibiotic is known, its target can be used as a screen for new antibiotics. Thus, if one is convinced that mucopeptide inhibitors represent the most effective and potentially least toxic type of antibiotic, it is possible to set up a screen to detect the producers of such antibiotics (Vasquez, 1960).

For the detection of antibiotics chromatographic and some physical methods are commonly used. Chromatographic identification of an antibiotic is mainly based on the solvent systems which are used. Physical methods for the identification of antibiotics comprise the techniques associated with the detection of luminescence in ultraviolet rays and absorption of ultraviolet radiation/radioactive tracing of antibiotics. The application of physical - chemical methods, for the identification of natural products has proven to be of great value (Nakayama, 1981). The present study showed that chromatography is one of a reliable techniques for the identification of antibiotics or their producer strains.

In chromatographic identification, the position of the spots of the test substances is very important to assess the results. The data showed that the $R_f$ value can be used to differentiate to a high
accuracy between groups of chemically similar antibiotics and also antibiotics inside such groups. If the R value is about 0.8 (and over) for the antibiotic under study in all the antibiotic under study in all the systems of the first type the alcoholic extract should be chromatographed in another system.

At present, the classification of antibiotic is based on their chemical structures. Although there exists great variation in its chemistry, yet there are certain similarities in their structures and it has been suggested that such antibiotics synthesized by to different organisms exert their therapeutic actions in a similar manner.

These polyene type of antibiotics inhibit the growth of a wide range of fungi, but less active against bacteria. The most characteristic physical properties of these polyene antibiotics is the ultraviolet absorption spectrum. The ultraviolet spectrum of all the polyene showed a regular series of sharp peaks of absorption which can be separated by sharp troughs, all in a range of 400-280 nm (Kabayashi and Medoff, 1977). Oroshnik and Mebane (1963) have given an extensive tabulation of the exact absorption maxima for many of the polyene antibiotics and
evaluated that the characteristic ultraviolet absorbing pattern is due to conjugated double bonds. Careful analysis of the ultraviolet spectra also indicates that there are several distinct classes of chromophores including tetraenes, pentaenes, hexaenes and heptaenes.

According to Waksman and Lechevalier (1962), the polyene antibiotics are active mainly against fungi. The antibiotics for which visible/ultraviolet light absorption reported are (i) Tetraenes: light absorption maxima (nm) at about 290 to 292, 300 to 305 and 317 to 320. (ii) Pentaenes: light absorption maxima (nm) at about 317 to 340, 330 to 340 and 349 to 358. (iii) Hexaenes: light absorption maxima (nm) at about 335 to 338, 335 to 359 and 373 to 380. (iv) Heptane: light absorption maxima (nm) at about 358 to 336, 377 to 388 and 399 to 410.

The polyene antibiotics exhibit characteristic ultraviolet absorption spectra typical of polyenic chromophores (Ball et al., 1957) are as follows:

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Polyene type</th>
<th>Absorption maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tetraene</td>
<td>290, 305, 318</td>
</tr>
<tr>
<td>2A</td>
<td>Pentaene</td>
<td>318, 333, 351</td>
</tr>
<tr>
<td>2B</td>
<td>Pentaene</td>
<td>325, 340, 358</td>
</tr>
<tr>
<td>3</td>
<td>Hexaene</td>
<td>340, 356, 378</td>
</tr>
<tr>
<td>4</td>
<td>Heptaene</td>
<td>360, 378, 405</td>
</tr>
</tbody>
</table>
Polyene having a conjugated polyene system are Nystatin, Amphotericin etc. Nastatin which is isolated from a strain of *Streptomyces noursei* is found to be useful in the treatment of gastrointestinal and local infections caused by *Candida albicans*.

Amphotericin is isolated from a strain of the species of *Streptomyces nodosus* is not one compound but consists of two closely related substances called amphotericin A and B. The compound B is more active and has been found to be most effective in systemic infections caused by *Candida albicans*. Another antifungal antibiotic which is not polyene is griseofulvin, which was first isolated from *Penicillium griseofulvum*.

When the data obtained in the ultraviolet absorption spectra study are compared with the absorption maxima of the known groups of polyene types, we find that *Streptomyces* strains Ac5, Ac40, Ac43, Ac52 and Ac56 showed maximum absorption spectrum in the present investigation, confirming the presence of polyene antibiotics. *Streptomyces* Ac1 and Ac54 showed absorption maxima at 270 nm in methanol, this seems to be a new type of antifungal antibiotic. It is also due to the fact that the UV light spectra of these compounds differ from polyene and non polyene antifungal antibiotic i.e., griseofulvin.
Most of the polyene antibiotics analyzed have certain common structural features in addition to a conjugated double bond system (Egorov, 1985).

Most of these antibiotics got degraded rapidly in the unsterilized soil. This can be attributed to the presence of active metabolites from other organisms which reacted with the antibiotics and also due to the absorption by the colloidal particles in the soil. In the present study the work was primarily directed against human pathogens particularly dermatophytes. Further the separation and isolation of different active components from different culture filtrates and their biochemical studies is in progress in collaboration with the workers in the department of chemistry.