



ANTIMICROBIAL SECONDARY METABOLITE-PRODUCING POTENTIAL OF STREPTOMYCES SP. ISOLATED FROM SOIL

MEERA KUMARI, NIRUPA KUMARI AND BIRENDRA PRASAD*

Microbial & Molecular Genetics Laboratory,
Department of Botany, Patna University, Patna-800 005, INDIA
*Corresponding Author (bprasad.pu@gmail.com)

A total of 150 strains of actinomycetes were isolated from different soil samples collected from various locations of Patna (Bihar). The actinomycetes strains were screened for their antimicrobial potential against test organisms such as *Escherichia coli* MTCC 739, *Staphylococcus aureus* MTCC 96, *Streptomyces lividans* TK 23 MTCC 4, and *Candida albicans* MTCC 227 by cross-streak method. The bioassay revealed that about 53.33% of actinomycetes isolates were active against test organisms. Characterization of these isolates has led to identification of unique strains of *Streptomyces* (MP 562 and NP 302) showing broad spectrum of antimicrobial properties. On the basis of λ_{max} values of culture filtrates, it has been suggested that these strains might be producing LL-E19085 and lipopeptide LY146032 like antibacterial and a flavone glycoside like antifungal antibiotics.

According to Bergey's Manual of Determinative Bacteriology, the strains MP 562 and NP 302 have been identified as *Streptomyces clavuligerus* MTCC 8722 and *Streptomyces anulatus* US7 MTCC 8724, respectively. Starch casein broth was found to be the best medium for the growth as well as antibiotic production for both strains. Maximum antimicrobial secondary metabolites production was achieved in late log phase which remained constant during stationary phase. The pH and temperature optima of antibiotics production have been found to be in the range of 6.5 to 7.0 and 30°C to 35°C, respectively.

Key words: Actinomycetes, Antimicrobial secondary metabolites, *Streptomyces clavuligerus*, *Streptomyces anulatus*

Streptomyces produces a remarkably diverse array of secondary metabolites, including many antibiotics (Gao et al. 2012). Actinomycetes are famous as producer of antibiotics and other biotechnological products (Challis and Hopwood 2003). The genus *Streptomyces*, which are common inhabitants of soil (Ravel et al. 2000) are especially prolific in this regard (Saadoun and Ghariabeh, 2003). They are gram positive filamentous bacteria (Oskay et al. 2004). Actinomycetes represent a rich source of biologically active metabolites such as antibiotics, agrochemicals, enzymes, immunosuppressants, antiparasitics and anticancer agents (Berdy 2005). Of these compounds, antibiotics predominate in therapeutic and commercial importance (Ouhdouch et al. 2001; Saadoun and Gharaibeh 2003). Investigation can possibly reveal actinomycetes species that produce novel antibiotics. It is anticipated that the isolation, characterization and the study on actinomycetes can be useful in the discovery of antibiotics and novel species of actinomycetes. Aim of the present study was

the isolation of actinomycetes from soil of Patna, Bihar, screening and characterization of antibiotic producer actinomycetes.

MATERIALS AND METHODS

Isolation of Actinomycetes Strains

Isolation of actinomycetes was performed by serial dilution spread plate technique using Starch Casein Agar Medium. The media containing per liter, 10.0g soluble starch; 0.3g casein; 2.0g KNO₃; 2.0g NaCl; 2.0g K₂HPO₄, traces of MgSO₄·7H₂O; CaCO₃; FeSO₄·7H₂O and 1.5% (w/v) agar, pH 6.8±0.2. 0.1g of soil samples was dissolved in 9.9 ml normal saline and serially diluted. 0.1ml of inoculum from desired dilution was spreaded on sterile and solidified Starch casein agar media containing plates. After incubation of 3-4 days at 35±10°C, colonies (rough and chalky) of actinomycetes were transferred from mixed culture of the plates on separate agar plates and incubated at 35±10°C for 7 days. After repeated restreaking on fresh media, finally the pure cultures were transferred to slant and preserved in refrigerator.

Table 1: Production of antibiotic in different media

Media	Test Organisms							
	Zone diameter of inhibition (in mm)							
	<i>Streptomyces lividans</i> MTCC4		<i>Staphylococcus aureus</i> MTCC96		<i>Escherichia coli</i> MTCC739		<i>Candida albicans</i> MTCC227	
	A	B	A	B	A	B	A	B
GAB	00	3	00	3	4	3	2	00
SCB	00	8	00	10	20	15	12	00
YEMEB	00	7	00	9	8	12	4	00
CzDB	00	2	00	2	4	3	2	00
NB	00	5	00	6	17	5	9	00
OMB	00	5	00	7	8	11	10	00
SB	00	6	00	7	9	10	5	00
SMB	00	7	00	8	18	10	10	00

A: *Streptomyces clavuligerus*; B: *Streptomyces anulatus*

GAB: Glycerol Asparagine Broth, SCB: Starch Casein Broth, YEMEB: Yeast Extract Malt Extract Broth, CzDB: Czapek Dox Broth, NB: Nutrient Broth, OMB: Oat Meal Broth, SB: Starch Broth and SMB: Soyabean Meal Broth

Table 2: Production of antibiotic at different pH

pH	Test Organisms							
	Zone diameter of inhibition (in mm)							
	<i>S. lividans</i> MTCC4		<i>S. aureus</i> MTCC96		<i>E. coli</i> MTCC739		<i>C. albicans</i> MTCC227	
	A	B	A	B	A	B	A	B
5.0	00	3	00	4	13	5	8	00
6.0	00	6	00	8	15	10	10	00
7.0	00	8	00	10	20	15	12	00
8.0	00	7	00	9	16	13	10	00
9.0	00	7	00	9	12	10	8	00
10.0	00	6	00	8	10	9	6	00

A: *Streptomyces clavuligerus*; B: *Streptomyces anulatus*

Table 3: Production of antibiotic at different temperature

Temperature	Test Organisms							
	Zone diameter of inhibition (in mm)							
	<i>S. lividans</i> MTCC4		<i>S. aureus</i> MTCC96		<i>E. coli</i> MTCC739		<i>C. albicans</i> MTCC227	
	A	B	A	B	A	B	A	B
25°C	00	2	00	4	3	6	5	00
30°C	00	5	00	7	18	10	10	00
35°C	00	8	00	10	20	15	12	00
42°C	00	0	00	0	16	0	8	00
50°C	00	0	00	0	0	0	0	00

A: *Streptomyces clavuligerus*; B: *Streptomyces anulatus*

The germination of the spores was observed within 24 hours of growth. The pattern of growth of both of the strains was found to be similar. It was observed that there was remarkable increase in dry weight of both isolates upto 14 days of incubation. In both strains growth patterns between 14 and 21 days were found to be very smooth with slight increase in the dry weight of the mycelia. The exponential phase lasted upto 21 days and then stationary phase was noticed (Figs.1 and 2).

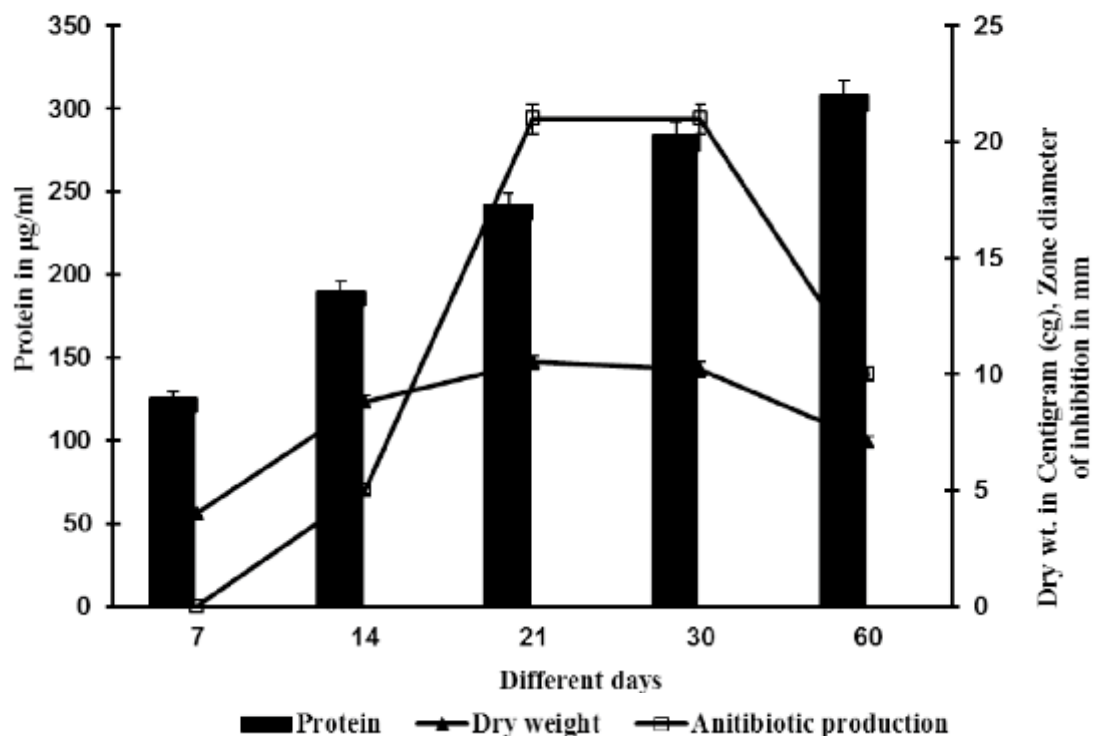


Figure 1. Time course showing relationship between dry weight, protein and antibiotic production by *Streptomyces clavuligerus*

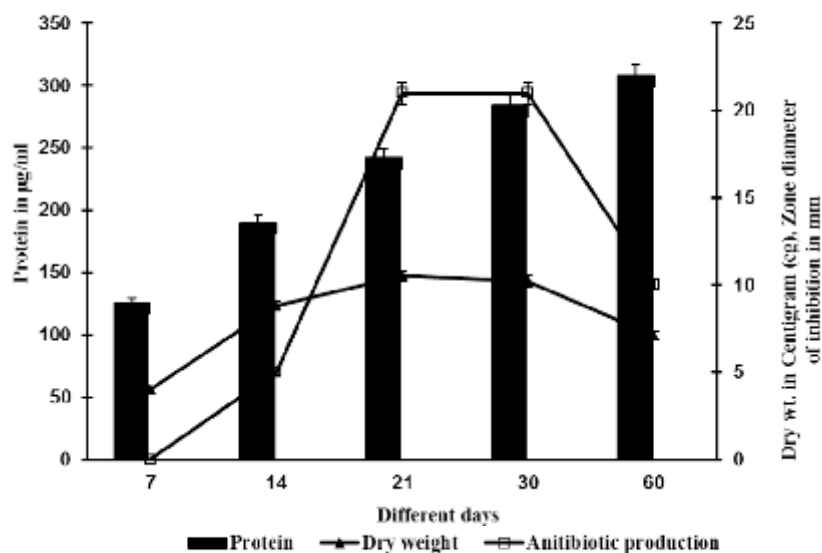


Figure 2. Time course showing relationship between dry weight, protein and antibiotic production by *Streptomyces anulatus* US7 MTCC 8724

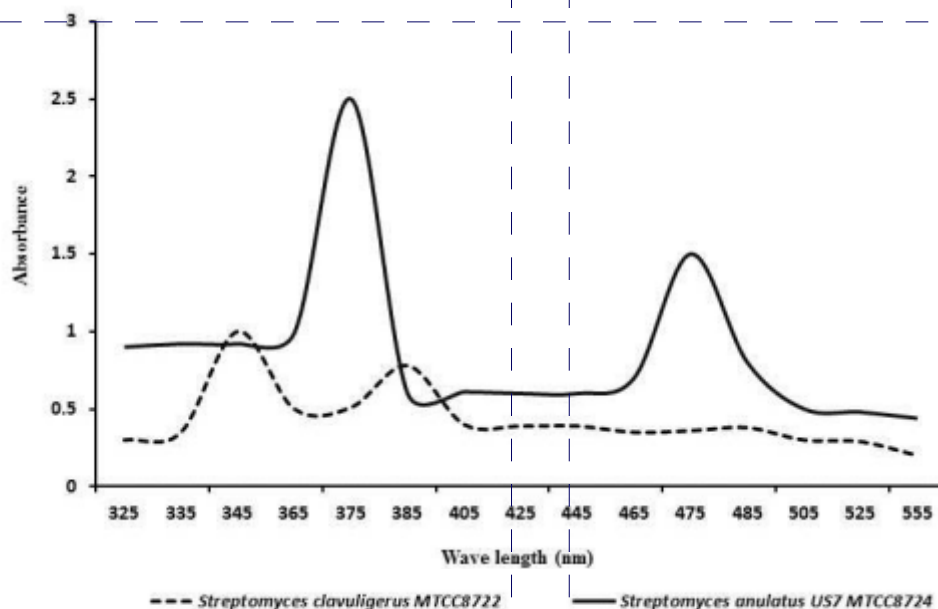


Figure 3. max of metabolites leaching in 60 days old cultural filtrates by *Streptomyces clavuligerus* MTCC 8722 and *Streptomyces anulatus* US7 MTCC 8724

Screening of Antibiotic Producing Actinomycetes

Screening of Actinomycetes for antimicrobial activity was performed by cross streak method (Waksman and Lechavalier 1962, Egorov 1985). The standard test organisms were *Streptomyces lividans* TK23 MTCC4, *Staphylococcus aureus* MTCC 96, *Escherichia coli* MTCC 739 and *Candida albicans* MTCC 227. All test organisms were obtained from MTCC & Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh.

Identification

The strains were characterized for taxonomic identification based on the parameters described in Bergey's manual of determinative bacteriology (Holt *et al.* 1993). Clonal culture of the strains was deposited in MTCC & Gene Bank, IMTECH, Chandigarh (India) with Accession Number *Streptomyces clavuligerus* MTCC 8722 and *Streptomyces anulatus* US7 MTCC 8724. The strains were also preserved in 20% glycerol at -20°C in cryovials as laboratory stock.

Preparation of Culture Filtrate

About 106 spores of each strain were inoculated in a 250 ml capacity conical flask

containing starch casein broth media. All inoculated flasks were incubated at stationary state at the temperature of 35±10°C for 7, 14, 21, 30 and 60 days. On particular day broth culture were filterated by suction pump on preweighed whatman filter paper No. 1. The filtrate were further centrifuged at 5000g for 15 minutes. Now, filtrate were used for further detection of antimicrobial properties and secondary metabolites.

Optimization of Condition for Antibiotic Production

To optimize the conditions for antibiotic production, strains were grown in eight different media (Glycerol Asparagine Broth, Starch Casein Broth, Yeast Extract Malt Extract Broth, Czapek Dox Broth, Nutrient Broth, Oat Meal Broth, Starch Broth and Soyabean Meal Broth) as well as at different pH range (5.0-10.0) and temperatures (25°C, 30°C, 35°C, 42°C and 50°C).

Estimation of Dry Weight

Liquid grown mycelia were harvested on dried (at 90°C for 24 hours) and preweighed Whatman filter paper No. 1 with the help of suction pump. Harvested mycelia were dried in the oven at 80°C for 24 hours. The difference

between the final and initial weights gave the dry weight of mycelia. The filtrates were kept for further experiment.

Estimation of Protein

Protein was estimated by the method suggested by Lowry *et al.* (1951). In 5 ml of alkaline solution C [50ml Solution A (2%Na₂CO₃ in 0.1N NaOH)+1ml Solution B (0.5% CuSO₄.5H₂O in 1% Sodium potassium tartrate)], 1ml of suitably diluted culture filtrate was added, mixed thoroughly and allowed to stand at room temperature for 10 minutes. Diluted (50%) Folin-Ciocalteu reagent (0.5ml) was added with gentle stirring. After 30 minutes, extinction was read at 750nm against the appropriate blank (5 ml alkaline solution C + 1 ml distilled water + 0.5 ml diluted Folin Ciocalteu reagent). The amount of protein was calculated by extrapolating with a standard curve prepared with the use Bovine Serum Albumine (BSA).

Antibiotic Producing Potential

Antibiotic producing potential of the strains was detected by well diffusion method on agar plate. The diameter of the zone of complete inhibition was measured to the nearest whole millimeter. Test organisms used for bio-assay were Gram positive (*Streptomyces lividans* TK23 MTCC4, *Staphylococcus aureus* MTCC 96), and Gram negative (*Escherichia coli* MTCC 739) bacteria and a fungus (*Candida albicans* MTCC 227).

Spectrophotometer Analysis of Culture Filtrate

Different biomolecules have different absorbance spectra in UV and visible light that enable the characterization of secondary metabolites. For determination of λ_{max} of biomolecules/secondary metabolites spectrophotometer (HITACHI-3210) was used during present investigation. Culture filtrate of different days grown culture was scanned over a range of wave length (200 - 900nm) against starch casein broth as a reference. On the basis of scanning graph, λ_{max} was determined.

RESULTS

A total of 150 strains of Actinomycetes were

isolated from 36 different soil samples from in and around of Patna, India. Screening of these isolates for antimicrobial properties revealed that about 53.33% of actinomycetes isolates were found active against at least one of the test organisms. Out of 150 isolates 12 isolates were found efficient antimicrobial metabolites producer. Two isolates having number MP562 and NP302 were found unique in inhibiting the growth of both Gram positive and Gram negative bacteria and a fungus. On the basis of morphological, physiological and biochemical characteristics MP562 and NP302 were identified as *Streptomyces clavuligerus* and *Streptomyces anulatus*, respectively and deposited in Microbial Type Culture Collection and Gene Bank, IMTECH, Chandigarh, India, with accession number MTCC 8722 and MTCC 8724 respectively.

The Starch casein broth was found to be the most suitable media for antibiotic production by both of the selected *Streptomyces* strains. Nutrient broth, Soyabean meal broth and Oat meal broth also favoured the production of antibiotics. In contrast, Glycerol asparagine broth and Czapek-dox broth media were able to produce very less amount of antibiotics. Yeast extract malt extract broth and Starch broth media showed moderate level of antibiotic production (Table-1).

Maximum antibiotic yield was obtained at pH 7.0. Antibiotic yields were low at low pH (5.0) as well as high pH (10.0) (Table-2).

During present investigation a temperature of 35°C was found to be the most suitable for antibiotic production by both of the strains of *Streptomyces*. No antibiotic production was recorded by any strains at 50°C. Zone diameter of inhibition was less at 30°C and 42°C. A very poor zone of inhibition appeared at 25°C (Table-3).

It was observed that the strains *Streptomyces clavuligerus* produced 300µg/ ml of protein after 60 days of growth. While *Streptomyces anulatus* produced 678µg /ml of proteins after 30 days of growth. Both the strains showed a significant increase in protein yield from 7 days

onwards (Figs.1 and 2).

Antibiotic production was not detected in 7 days old culture filtrate. It was noticed after 14 days of incubation. *Streptomyces clavuligerus* showed maximum antibiotic production in 30 days old culture filtrates which remained constant for stationary phase while *Streptomyces anulatus* showed maximum antibiotic production in 60 days old culture filtrates (Figs.1 and 2).

Streptomyces anulatus showed antagonistic activities against Gram+ve and Gram-ve bacteria. In contrast *Streptomyces clavuligerus* exhibited antifungal activity including antibacterial property.

During present investigation absorption spectrum of culture filtrate of *Streptomyces clavuligerus* showed two peaks at 345nm and 385nm. An increasing trend in the absorbance (O.D) values was observed in different days culture filtrates (upto 30 days of incubation) thereafter a decrease in the absorbance value was observed. Maximum O.D values were found 0.99 and 0.78 at 345nm and 385nm, respectively (Fig. 3). Pigmented culture filtrate of *Streptomyces anulatus* also showed two peaks at 375 nm and 475 nm. An increasing trend in the absorbance (O.D) value was observed in different days culture filtrates. Maximum OD values of 2.32 and 1.48 were observed at 375nm and 475nm respectively in 60 days old culture filtrate (Fig. 3).

DISCUSSION AND CONCLUSION

The methods employed for isolation of actinomycetes strains have been found to be common with the methods of earlier workers (Williams and Wellington 1982,). Numbers of colonies of actinomycetes have been found to be highest on Starch casein agar medium which is considered to be one of the best medium for isolation of actinomycetes (Barnett 1993, Singh and Agrawal 2003).

Streptomyces spp. has the exceptional ability to produce a broad range of low molecular weight antibiotics and other secondary metabolites (Hodgson 2000, Watve *et al.* 2000). Many of these compounds have antibacterial and

antifungal properties and are used as therapeutic agents in medicine and agriculture (Fulgueira *et al.* 2004). Pigment production is another unique aspect of these organisms (Krassilnikov, 1981).

Environmental factors, such as carbon and nitrogen sources, temperature, pH and method of cultivation can affect the timing and extent of production of antibiotic, as has been found for many other secondary metabolites (Martin and Demain, 1980)). Amongst various nutritional requirements, carbon source and nitrogen source are generally regarded as important factors of metabolism, and several examples of the production of metabolites in media with optimized contents of these components are also described in the literature (Silva *et al.* 2012; Khopade *et al.* 2012). In majority of *Streptomyces*, the preferred carbon source is starch, especially for the production of secondary metabolites (Syed *et al.* 2009; Vijayabharathi *et al.* 2012). Production of antifungal metabolite has been known to be influenced by media components and cultural conditions such as aeration, pH and temperature (Iwai and Omura 1982). Deviation from optimum temperature severely affects the yield of antifungal metabolites (Yoshida *et al.* 1972). Optimum temperature for growth and antibiotic production was observed at $35 \pm 10^\circ\text{C}$. The isolate *Streptomyces rochei* AK 39 exhibited the similar result against the dermatophytes when grown on SCA medium. pH is a significant factor that influences the physiology of a microorganism by affecting nutrient solubility and uptake, enzyme activity, cell membrane morphology, by product formation and oxidative reduction reactions (Bajaj *et al.* 2009). Evaluation of data at different pH conditions indicated that pH 7.0 is suitable for growth and maximum production of antibiotics for both the strains.

Actinomycetes species are heterotrophic feeders and they can utilize both simple and complex molecules as nutrients. They have characteristic biological aspects such as mycelial form of growth that culminates in sporulation and the ability to produce wide

varieties of secondary metabolites including most of the antibiotics (Schneider *et al.* 1993; Marines *et al.* 1994; Qin *et al.* 1998). An intermediate metabolite from primary metabolism serves as precursor for the biosynthesis of antibiotics. Therefore, the growth profile, closely connected with the metabolic capacities of the producing organism, greatly influences the biosynthesis of antibiotic. It has been suggested by Augustine *et al.* (2005) that the maximum production of metabolite occurs in late log phase and remains constant during stationary phase. *Streptomyces clavuligerus* showed an increase in biomass upto 21 days of incubation (late log phase). Maximum yield of antimicrobial metabolites was observed in late log phase which remained constant during stationary phase (upto 30 days) indicating the direct relationship between growth rate and metabolite production. Results showed by *S. anulatus* also supported above mentioned views.

This could be because the organism might have reached death phase. Usually synthesis of the secondary metabolites occurs after metabolite synthesis process.

Exponential growth continues until limited through depletion of essential nutrients, occupancy of all spatial niches, or the accumulation staling compounds (Allan and Prosser 1987). With the exception of *S. roseosporus* cultures which formed spores in a complex medium that was not manipulated to ensure nutrient deprivation (Huber *et al.* 1987), the physiological condition most often associated with spore formation is nutritional downshift. Down shifts resulted from depletion of essential nutrients, in most cases nitrogen or phosphate (Kendrick and Ensign 1983, Koepsel and Ensign 1984). Nutrient limitation in *Streptomyces* has been shown to trigger differentiation and induction of secondary metabolism (Brana and Demain 1988). The major processes associated with the phase of secondary metabolism in *Streptomyces thermoviolaceus* consists of antibiotic synthesis and protein secretion. These occur at

a time when nutrients in growth media are still in excess and growth is still occurring (James and Edwards 1989). Our present findings in case of *Streptomyces clavuligerus* also support the above mentioned views.

It has also been suggested that the protein secretion is invariably linked to the production of granitacin antibiotic in *Streptomyces thermoviolaceus*. Our findings also suggested a distinct relationship between extracellular protein and antibiotic production. *Streptomyces clavuligerus* showed continuous increase in amount of protein in broth upto 60 days of incubation whereas decline in antibiotic production was recorded during 30 to 60 days of incubation. Continuous increase in the amount of antibiotic production upto 60 days of incubation had been observed in the case of *Streptomyces anulatus*, whereas there was decline in protein yield after 30 days of incubation. The reason might be the conversion of one biomolecules as precursor of another biomolecules.

Absorption spectra analyses of culture filtrates were performed by UV-VIS spectrophotometer in order to identify the metabolites. The culture filtrates of the strains *Streptomyces clavuligerus* MTCC 8722 and *Streptomyces anulatus* MTCC 8724 exhibited two peaks. Similar observation has also been reported in with *Streptomyces coelicolor* which produces two antibiotics, a blue coloured actinorhodin (ACT) and a red coloured undecyprodiginines (Ryu *et al.* 2006). Krassilnikov (1981) has also reported that the actinomycetes often produce two or more antibiotics simultaneously having different natures. The first peak (345nm) of *Streptomyces clavuligerus* was most likely to be an antifungal antibiotic flavone glycoside (347 nm) like compounds (Jensen *et al.* 1998). The second peak of *Streptomyces clavuligerus* was at 385nm which is the λ_{max} of antibacterial antibiotic LL-E 19085 beta and gamma (Carter *et al.* 1992). The second peak (475nm) of *Streptomyces anulatus* spectra was very close to 465nm of lipopeptide antibiotic LY146032 (Lakey and Ptakey 1988).

It has been observed that in *Streptomyces*

clavuligerus the absorbance values increased upto 30 days of incubation. In *Streptomyces anulatus* there is increase in absorbance value upto 60 days of incubation. This increase of absorbance values might be due to constitutive production of secondary metabolites. But in *Streptomyces clavuligerus* after 30 days of incubation, it decreased. It might be due to feedback inhibition by secondary metabolites (Zahn *et al.* 2001).

According to Lancini *et al.* (1995), the activity of an antibiotic is defined and measured in terms of its ability to inhibit microbial growth (bacteria, fungi and protozoa). In the present study the strains *Streptomyces clavuligerus* and *Streptomyces anulatus* showed broad antimicrobial spectra, as they inhibited the Gram-positive, Gram-negative bacteria as well as fungi. The significant results make these strains attractive for further investigation and industrial exploitation.

REFERENCES

- Allan E J & Prosser J I 1987 Colony growth of *Streptomyces coelicolor* A 3(2) under conditions of continuous nutrient supply FEMS Microbiol Lett **43** 139–142.
- Augustine S K, Bhavsar S P & Kapadnis B P 2005 Production of a growth dependent metabolite active against dermatophytes by *Streptomyces rochei* AK 39. Indian J Med Res **121** 164–170.
- Berdy J 2005 Bioactive microbial metabolites, a personal view. J Antibiotics **58** 1–26.
- Barnett M E 1993 Microbiology laboratory exercise to accompany foundation in Microbiology. W.M.C. Brown Publishers Dubuque.
- Bajaj I B, Lele S S & Singhal R S 2009 A statistical approach to optimization of fermentative production of poly(c-glutamic acid) from *Bacillus licheniformis* NCIM2324 Bioresour Technol **100** 826–832.
- Brana A F & Demain A L 1988 Nitrogen source control of antibiotic biosynthesis in actinomycetes. In: Nitrogen source control of microbial processes pp. 99–119. Edited by S. Sanchez Esquivel Boce Raton, Florida CRC Press.
- Carter G T (Suffern NY, US) Goodman J J (Spring valley, NY, US) & Labeda D P (Peonia, IL, US). 17 March 1992 Antibiotic LL-E19085. United States Patent 5096907.
- Challis G L & Hopwood D A 2003 Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* spp. Proc Natl Acad Sci US **100** 14516–14555.
- Egorov N S 1985 Antibiotics: A Scientific Approach. Mir publishers, Moscow. Pp440
- Fulgueira C L, Amiglot S L & Magni C 2004 Growth inhibition of toxogenic fungi by a proteinaceous compound from *Streptomyces* spp. C/33-6 Current Microbiology **48** 135–139.
- Gao C, Hindra M D, Yin C & Elliott MA 2012 Crp is a Global Regulator of Antibiotic Production in *Streptomyces*. Bio **3** 407–412.
- Holt J G, Krieg N R, Sneath P H, A Staley J T & Williams S T 1993 Bergey's Manual of Determinative Bacteriology 9th Edition. Williams & Wilkins 605–609.
- Hodgson D A 2000 Primary metabolism and its control in Streptomycetes: a most unusual group of bacteria Adv Microb Physiol **42** 47–238.
- Huber F M, Pieper R L & Mertz F P 1987 Sporulation of *Streptomyces roseosporus* in submerged culture J Indust Microbiol **2** 235–241.
- Iwai Y & Omura S 1982 Culture conditions for screening of new antibiotics J Antibiot **35** 123–141.
- James P D A & Edwards C 1989 The effect of temperature on growth and production of the antibiotic granaticin by thermotolerant Streptomycete J Gen Microbiol **135** 1997–2003.
- Jensen P R, Jenkins K M, Porter D & Fenical W 1998 Evidence that a new antibiotic flavones glycoside chemically defends the sea grass *Thalassia testudinum* against Zoosporic fungi. Appl Environ Microbiol **64** 1490–1496.
- Kendrick K E & Ensign J C 1983 Sporulation of *Streptomyces griseus* in submerged cultures J Bacteriol **155** 357–366.
- Koepsel R & Ensign J C 1984 Microcycle sporulation of *Streptomyces vridochromogens* Archives Microbiol **140** 9–14.
- Khopade A, Ren B, Xiang-Yang Liu XY, Mahadik K, Zhang L & Kokare C 2012 Production and characterization of biosurfactant from marine *Streptomyces* species J Colloid Interface Sci. **367** 311–318.
- Krassilnikov N A 1981 Ray Fungi Vol 1 Amerind Publishing Co Pvt Ltd, New Delhi Pp265.
- Lancini G, Parenti F & Gallo G G 1995 Antibiotics-A multidisciplinary approach Plenum Publishing Corporation New York, New York.
- Lakey J H & Ptak M 1988 Fluorescence indicates a calcium-dependent interaction between the lipopeptide antibiotic LY 146032 and Phospholipid membranes. Biochem **28** 4639–4645.
- Lowry O H, Rosenbrough N J, Farr A L & Randell R J 1951 Protein measurement with the Folin Phenol reagent J Biol Chem **193** 265–275.
- Martin J F & Demain A L 1980 Control of antibiotic biosynthesis Microbiol Rev **44** 230–251.
- Marines M, Costa S O & Padilla G G 1994 Effects of genotoxic agents on his⁺ revertants and survival of spores of *Streptomyces aureofaciens* World. J Microbiol Biotechnol **10** 560–562.

- Oskay M, Tamer U A & Azeri C 2004 Antibacterial activity of some actinomycetes isolated from farming soils of Turkey. *African J Biotechnol* **3** 441-446.
- Ouhdouch Y, Barakate M & Finanse C 2001 Actinomycetes of Moroccan habitats: Isolation and screening for antifungal activities. *Eur J Soil Biol* **37** 69-74.
- Qin Z, Zhou X, Zhou Q, Chen H, Hopwood D, Kieser T & Denz Z 1998 Strain improvement by genetic manipulation for the overproduction of several chemically unrelated antifungal antibiotics which are used for plant protection. *Abster VIII Int Symp Gen Ind Microorg* Jerusalem Israel 50.
- Ravel J E, Wellington M H & Hill R T 2000 Interspecific transfer of *Streptomyces* giant linear plasmids in sterile amended soil microcosms. *Appl Environ Microbiol* **66** 529-534.
- Ryu Y G, Butter M J, Chater K F & Lee K J 2006 Engineering of primary carbohydrate metabolism for increase production of Actinorhodin in *Streptomyces coelicolor*. *Appl Environ Microbiol* **72** 7132-7139.
- Saadoun I & Gharaibeh R 2003 The *Streptomyces* flora of Badia region of Jordan and its potential as a source of antibiotics active against antibiotic-resistant bacteria. *J Arid Environ* **53** 365-371.
- Schneider D, Aigle B, Leblond P, Simonet J M & Decaris D 1993 Analysis of genome instability in *Streptomyces ambofaciens*. *J Gen Microbiol* **139** 2559-2567.
- Singh D & Agrawal V P 2003 Diversity of Actinomycetes of Lobuche in Mount Everest *Proceedings of International Seminar on Mountains-Kathmandu* Pp 357-360.
- Silva I R, Kassawara M, Martins M K, Carvalho C M, Azevedo J L & Silva R E L P 2012 The Effect of varying culture conditions on the production of antibiotics by *Streptomyces* spp., Isolated from the Amazonian Soil *Ferment Technol* **1** 1000105-1000110.
- Syed D G, Lee J C, Li W J, Kim C J & Agasar D 2009 Production, characterization and application of keratinase from *Streptomyces gulbargensis*. *Bioresour Technol* **100** 1868-1871.
- Vijayabharathi R, Devi P B, Sathyabama S, Bruheim P, Priyadarisini V B 2012 Optimization of resistomycin production purified from *Streptomyces aurantiacus* AAA5 using response surface methodology. *J Biochem Tech* **3** 402-408.
- Waksman S A & Lechevalier H A 1962 *The actinomycetes* volume-III, The Williams and Wilkins Company, Baltimore USA Pp 20.
- Wathe M G, Tickoo R, Jog M M & Bhole B D 2001 How many antibiotics are produced by the genus *Streptomyces*. *Arch of Microbiol* **176** 386-390.
- Williams S T & Wellington E M H 1982 *Principles and Problems of selective isolation of microbes in bioactive metabolic products: Search and discovery* (Bu'lock J D, Nisbet L J & Winstanley D J Ed) Academic Press London, Pp 9-26.
- Yoshida N, Tani Y & Ogata T C 1972 Cryomycin - A new peptide antibiotic produced only at low temperature. *J Antibiot* **25** 653-659.
- Zahn J A, Higgs R E & Hilton M D 2001 Use of direct-infusion electrospray mass spectrometry to guide empirical development of improved conditions for expression of secondary metabolites from Actinomycetes. *Appl Environ Microbiol* **67** 377-386.