

## Identification and Characterization of Antibiotic-Producing Actinomycetes Isolates

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### ABSTRACT

Actinomycetes are a gram-positive, filamentous subgroup of bacteria most known for antibiotic production. In fact, most of the antibiotics available today have originated from actinomycetes, namely from the genus *Streptomyces*. Novel bacteria with antimicrobial activities have been discovered from bacterial screen studies for decades and there is still much more yet to be unearthed. One hundred seventy five strains of actinomycetes were isolated from 38 different soil samples from different locations in Patna, India. Potential antibiotic producers were screened against four test microorganisms (*Escherichia coli* MTCC 739, *Staphylococcus aureus* MTCC 96, *Streptomyces lividans* TK23 MTCC 4 and *Candida albicans* MTCC 227). The bioassay revealed that about 26% of actinomycetes isolates were active against at least one of the test microorganism. Characterization of a selected isolate has led to identification of a unique strain of actinomycetes (MP 525) showing broad-spectrum antibacterial and antifungal properties. The strain MP 525 has been morphologically characterized as *Streptomyces* sp. and deposited at MTCC, Chandigarh, India with accession number 8723. The 16S rRNA gene of the strain *Streptomyces* sp. US7 MTCC 8723 was sequenced and the DNA sequence was deposited at NCBI, Bethesda (GenBank accession. No. HQ659005). On the basis of  $\lambda_{max}$  values of culture filtrates, it has been suggested that the strain might be producing LL-E19085-like antibacterial and a flavone glycoside-like antifungal antibiotics, which can be further exploited for industrial and biological applications.

**Keywords:** Actinomycetes, *Streptomyces*, Antibiotic Production, Phylogenetic Tree

### 1. INTRODUCTION

The phylum Actinobacteria contains several hundred bacterial species that are commonly found in soil, freshwater and marine environments. Some Actinobacteria are also animal and plant pathogens, such as *Mycobacterium*, *Corynebacterium*, *Nocardia*, *Rhodococcus* and a few species of *Streptomyces*. These species are gram-positive filamentous bacteria, which produce aerial hyphae and differentiate into chains of spores (Oskay *et al.*, 2004).

Actinobacteria are commonly known as producers of antibiotics and other metabolites (Challis and Hopwood, 2003). In fact, 70-80% of the commercially available secondary metabolites have been isolated and

characterized from several species of actinomycetes (Khanna *et al.*, 2011). These secondary metabolites represent an opulent source of biologically active compounds such as antibiotics, agrochemicals, enzyme, immunosuppressants, antiparasitics and anticancer agents (Berdy, 2005). However, over the last decade, the search for antibiotics and other metabolites in actinomycetes and other microorganisms has become less popular due to the diminishing success of discovering novel strains (Baltz, 2008). As a result, screening for potential chemicals increased but did not yield much success, which is not surprising since Actinobacteria have evolved their capabilities over a billion years (Baltz, 2008). A mathematical model designed to estimate the number of undiscovered

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antimicrobial agents from the genus *Streptomyces* predicts that the decline of finding such agents is due to the declining efforts in screening more microorganisms and not due to a lesser number of undiscovered antimicrobial agents (Watve *et al.*, 2001) and thus, the search for microorganisms and new antibiotics has gained importance. For example, it was discovered in Egypt that a strain of actinomycetes, *Streptomyces antibioticus* AZ-Z710, produced a strong antifungal antibiotics (Atta, 2010). Furthermore, there is an active search in Turkey for an antibacterial agent, producing *Streptomyces* sp. that can fight antibiotic resistant bacteria (Ceylan *et al.*, 2008) and in China, a new strain of *Streptomyces* was discovered that kills certain pathogenic fungi (Jiang *et al.*, 2007). Thus, it is anticipated that the isolation and characterization of actinomycetes from different environments can be useful in the discovery of novel strains as well as antibiotics.

The goal of the present study was to isolate actinomycetes from soils from different localities in Patna, India and to screen for potential antibiotic producers from varied environments. After an initial screen for antimicrobial activity, isolates were then morphologically characterized using the parameters described in Bergey's Manual of Determinative Bacteriology (Holt, 1994). To identify the strain, 16S rRNA gene from the newly isolated strain was compared against the NCBI database for closely related 16S rRNA gene sequences. Phylogenetic analysis confirmed the taxonomic status of the new strain as *Streptomyces* and the organism was classified as *Streptomyces* sp. US7 MTCC 8723.

## 2. MATERIALS AND METHODS

### 2.1. Isolation of Strains and Antibiotics

Single colonies of actinomycetes were isolated by serial dilution and spread-plate method using Starch Casein Agar (SCA) Medium (1 L solution of 10.0 g soluble starch; 0.3 g casein; 2.0 g KNO<sub>3</sub>; 2.0 g NaCl; 2.0 g K<sub>2</sub>HPO<sub>4</sub>; traces of MgSO<sub>4</sub>·7H<sub>2</sub>O; CaCO<sub>3</sub>; FeSO<sub>4</sub>·7H<sub>2</sub>O and 1.5 g agar, pH 6.8±0.2). Soil samples (0.1 gram) were suspended in 9.9 ml normal saline (0.87% NaCl, w/v) and serially diluted. 0.1 mL of inoculums from desired dilution was spread onto sterile SCA agar plate. After incubation at 35 ± 1°C for 3-4 days, rough and chalky colonies were further streaked out onto new plates. The pure cultures were then transferred to SCA slants for further storage.

Screening of single colonies for antimicrobial activity was performed by cross streak method (Waksman and Lechevalier, 1962) using four test microorganisms (*Streptomyces lividans* TK23 MTCC4, *Staphylococcus*

*aureus* MTCC 96, *Escherichia coli* MTCC 739 and *Candida albicans* MTCC 227). All test microorganisms were procured from Microbial Type Culture Collection (MTCC) and GeneBank, Institute of Microbial Technology (IMTECH), Chandigarh, India. The isolates were characterized for taxonomic identification based on the parameters described in Bergey's Manual of Determinative Bacteriology (Holt, 1994). Furthermore, the selected strains were stored in 20% glycerol, at -20°C, in cryovials, as laboratory stock.

### 2.2. Phylogenetics Analysis

The 16S rRNA gene was amplified from *Streptomyces* sp. US7 MTCC 8723 strain and the subsequent PCR product was sequenced. The sequence was deposited at NCBI, Bethesda. The 16S rRNA gene sequences of closely related taxa were retrieved from the GeneBank database using BLASTN (Altschul *et al.*, 1997). The MEGA 4.1 software was used for construction of phylogenetic trees that are optimal according to the Neighbor-Joining (NJ) method (Tamura *et al.*, 2007).

### 2.3. Preparing Culture Filtrate

About 10<sup>6</sup> spores of the strain were inoculated in a 250 mL capacity conical flask containing 60 mL Starch Casein Broth (SCB) media. All inoculated cultures were incubated within a stationary incubator at 35±1°C for 7, 14, 21, 30 and 60 days. The broth cultures were then filtrated by suction pump on preweighed Whatman filter paper no. 1. The resulting filtrates were centrifuged at 5,000×g for 15 min and supernatant was further characterized for antimicrobial activity.

### 2.4. Optimization of Condition for Antibiotic Production

To optimize the conditions for antibiotic production, the selected strain was grown in eight different media, Glycerol Asparagine Broth (GAB), Starch Casein Broth (GSB), Yeast Extract Malt Extract Broth (YMB), Czapek Dox Broth (CDB), Nutrient Broth (NB), Oat Meal Broth (OMB), Starch Broth (SB) and Soyabean Meal Broth (SMB) as well as at different pH ranges (5.0-10.0) and different temperature (25, 30, 35, 42 and 50°C).

### 2.5. Estimation of Dry Weight

Liquid grown mycelia were harvested and dried (at 90°C for 24 h) on preweighed Whatman filter paper no. 1 with the help of suction pump. Harvested mycelia were dried in the oven at 80°C for 24 h. The difference between the final and initial weight provided the dry weight of mycelia.

## 2.6. Protein Quantification

Protein amounts in the samples were estimated by using the Lowry *et al.* (1951) method. Bovine Serum Albumin (BSA) is used as standard.

## 2.7. Antibiotic Producing Potential

The antibiotic producing potential of the selected strain was determined by well diffusion method on an agar plate. The diameter of the zone of complete inhibition was measured to the nearest millimeter. Test organisms used for bioassay were Gram-positive bacteria (*Streptomyces lividans* TK23 MTCC4, *Staphylococcus aureus* MTCC96) and Gram-negative bacteria (*Escherichia coli* MTCC739) and a fungus (*Candida albicans* MTCC227).

## 2.8. Spectrophotometer Analysis of Culture Filtrate

Different bioactive compounds have different absorbance spectra in UV and visible light that enable the characterization of secondary metabolites.  $\lambda_{max}$  of secondary metabolites was determined by spectrophotometer (HITACHI-3210). The culture filtrates were scanned over a range of wavelength (200-900 nm) against Starch Casein Broth (SCB) as a reference. On the basis of the scanning graph,  $\lambda_{max}$  was determined.

## 3. RESULTS

### 3.1. Antibiotic production in Different Growth Media at Varying pHs and Temperatures

Antibiotic activity is defined and measured in terms of its ability to inhibit microbial growth of bacteria, fungi and protozoa (Lancini *et al.*, 1995). Screening of the isolates for antimicrobial properties revealed that about 33% of actinomycetes isolates in the present study were active against at least one of the four test microorganisms (*Streptomyces lividans* TK23 MTCC4, *Staphylococcus aureus* MTCC96, *Escherichia coli* MTCC739 and *Candida albicans* MTCC227). Of the total, 12 isolates were found to be efficient antimicrobial metabolites producers. One isolate, MP 525, was able to inhibit the growth of all four test organisms (*S. lividans*, *S. aureus*, *E. coli*) and *C. albicans*.

The level of antibiotic production in different growth media was listed in **Table 1**. Results revealed that Starch Casein Broth (SCB) was found to be the most suitable media for antibiotic production. Nutrient Broth (NB), Soybean meal broth (SNB) and Oat Meal Broth (OMB)

also favored the production of antibiotics. Yeast and Malt extract Broth (YMB) and Starch Broth (SB) media showed moderate level of antibiotic production. In contrast, Glycerol Asparagine Broth (GAB) and Czapek-Dox Broth (CDB) media were able to produce much less antibiotics.

In addition, antibiotic yield varies at different pH and temperatures as shown in **Table 2 and 3**, respectively. The maximum antibiotic production was observed in the cultures grown at pH 7.0 (**Table 2**) as well as at 35°C temperature (**Table 3**). No antibiotic production was detected at 50°C. Zone of inhibitions were small at 30 and 42°C and even smaller at 25°C (**Table 3**). Antibiotic production was not detected in 7-day-old culture filtrate, but was noticed after 14 days of incubation. It showed maximum antibiotic production in 30-day-old culture filtrates, which remained constant at stationary phase. *Streptomyces* sp. US7 MTCC8723 showed significant antagonistic activities against gram-positive bacteria and fungi.

**Table 1.** Production of antibiotics by actinomycete strain MP525 in different media, shown by the zone diameter (mm) of inhibition against each of the test organism

Media	<i>Streptomyces lividans</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Candida albicans</i>
	MTCC4	MTCC96	MTCC739	MTCC227
GAB	1	2	3	1
SCB	10	20	20	24
YEMEB	4	7	6	4
CzDB	0	0	2	2
NB	9	13	12	8
OMB	8	12	12	8
SB	5	8	7	5
SMB	9	10	12	8

**Table 2.** Production of antibiotic by actinomycete strain MP525 at different pH, shown by the zone diameter (mm) of inhibition against each of the test organism

pH	<i>Streptomyces lividans</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Candida albicans</i>
	MTCC4	MTCC96	MTCC739	MTCC227
5	10	8	8	3
6	18	18	17	7
7	20	20	22	8
8	19	19	17	7
9	15	14	14	6
10	12	12	10	4

**Table 3.** Production of antibiotics by actinomycete strain MP525 at different temperatures, shown by the zone diameter (mm) of inhibition against each of the test organism

Temp	<i>Streptomyces lividans</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Candida albicans</i>
	MTCC4	MTCC96	MTCC739	MTCC227
25°C	2	1	2	3
30°C	6	12	14	16
35°C	10	20	20	24
42°C	8	15	15	20
50°C	0	0	0	0

As shown in Fig. 1, spore germination was observed within 24 h of growth and there was remarkable increase in dry weight up to 14 days of incubation. Growth patterns between 14 and 21 days were found to be very even with slight increase in the dry weight of the mycelia. The exponential phase lasted for up to 21 days before reaching stationary phase. The strain showed a significant increase in protein yield from 7 days onwards with a maximum of  $560\mu\text{g mL}^{-1}$  after 60 days of growth.

### 3.2. Characteristic Absorption Spectra

Absorption spectral analysis of bacterial culture filtrates predicts the nature of the metabolites as an indicator of the specific bacterial species. In this investigation, the absorption spectrum of the culture filtrate of *Streptomyces* sp. US7 MTCC 8723 displayed two major peaks, one at 345 nm and the other at 390 nm, as shown in Fig. 2. An increasing trend in the absorbance (O.D) values was observed in different day culture filtrates (up to 30 days of incubation) and a decrease in the absorbance value was observed thereafter.

### 3.3. Phylogenetic Analysis of 16S Ribosomal RNA Genes and New Strain Identification

On the basis of morphological, physiological and biochemical characteristics, according to the Bergey's Manual of Determinative Bacteriology (Holt, 1994), MP 525 was identified as *Streptomyces* sp. US7 and deposited at Microbial Type Culture Collection and GeneBank, IMTECH, Chandigarh, India, with accession number MTCC 8723. The 16S rRNA gene sequence of the strain *Streptomyces* sp. US7 MTCC 8723 was deposited at NCBI, with a GenBank accession No. HQ659005. The 16S rRNA gene sequences from strains closely related to *Streptomyces* sp. US7 MTCC 8723 were retrieved from the GeneBank database using BLASTN (Altschul *et al.*, 1997). The Phylogenetic tree shown in Fig. 3 further confirmed that the newly identified strain belongs to the *Streptomyces* sp. In conclusion, the newly isolated strain *Streptomyces* sp. US7 MTCC8723 showed broad antimicrobial spectra as it inhibited Gram-positive bacteria, Gram-negative bacteria as well as fungi. These significant results make the strain suitable for further investigation and industrial exploitation.

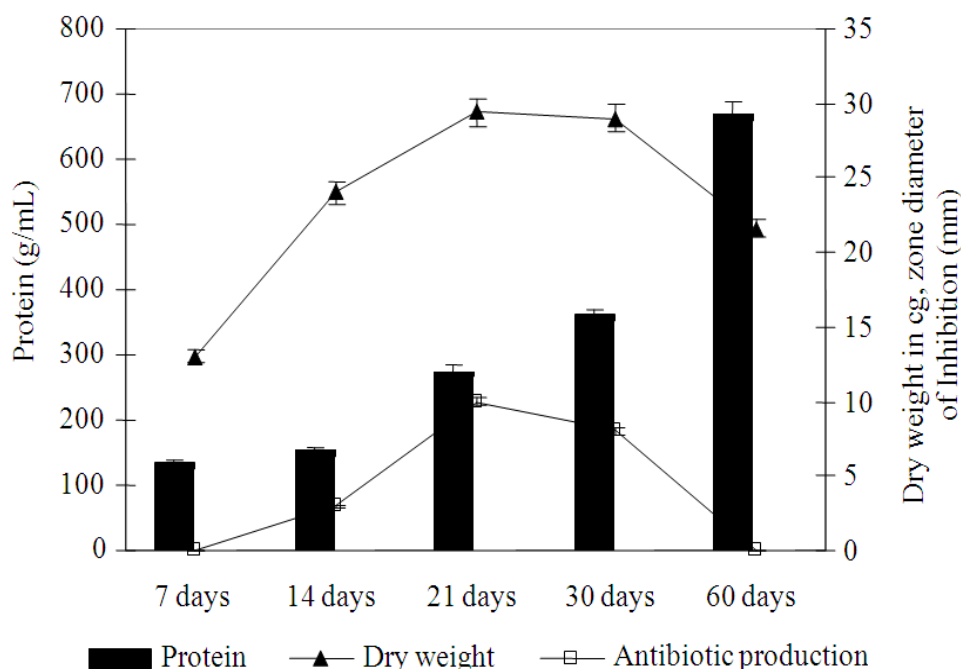
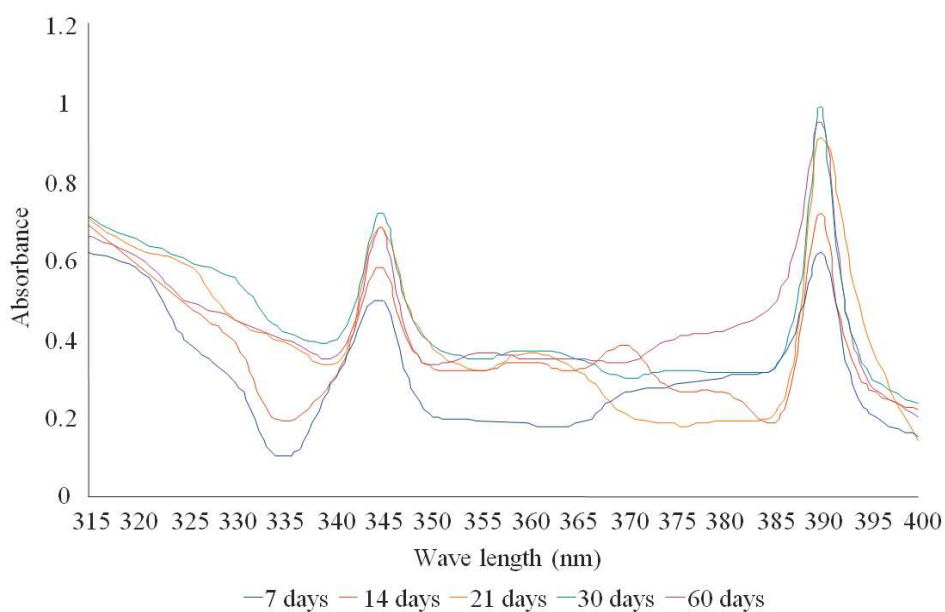
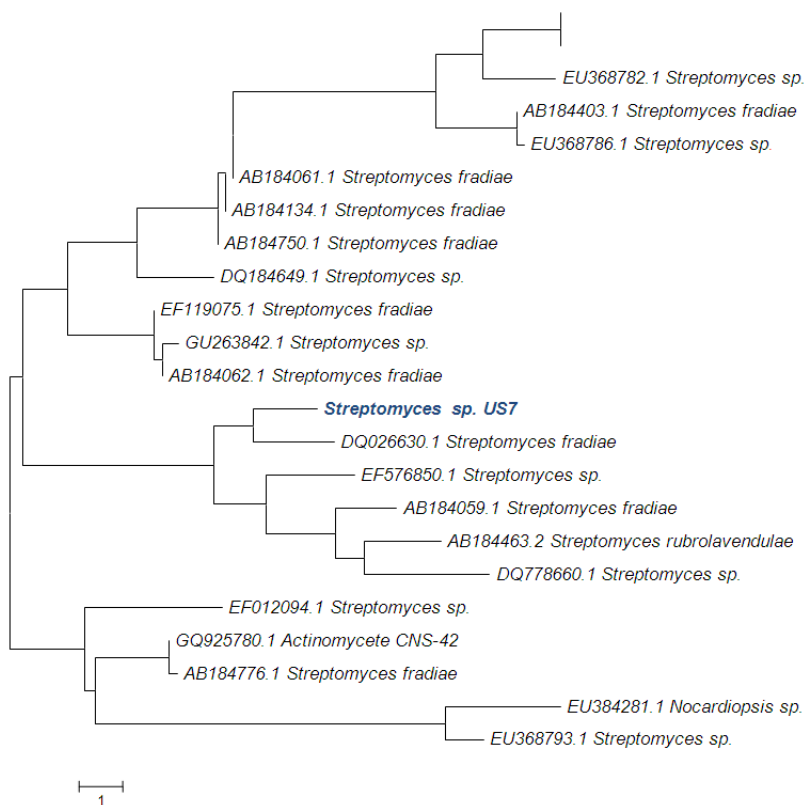


Fig. 1. Time course showing relationship between dry weight, protein and antibiotic production by *Streptomyces* sp. US7 MTCC 8723



**Fig. 2.** Absorption spectra of different days old culture filtrates of *Streptomyces* sp. US7 MTCC 8723



**Fig. 3.** Phylogenetic tree showing the evolutionary relationship of *Streptomyces* sp. US7 MTCC8723 with the other 22 taxa

#### 4. DISCUSSION

A total of 175 strains of actinomycetes were isolated from 38 different soil samples collected from different locations of Patna, India. These sites represented heterogeneous environments. In this study, the methods used for isolation of actinomycete strains were similar to methods that have been employed in earlier studies (Williams and Wellington, 1982). The number of bacterial colonies was highest on the SCA medium, which is known as the optimal growth media for isolation of actinomycetes (Hsu and Lockwood, 1975).

Absorption spectral analysis of bacterial culture filtrates predicts the nature of the metabolites as an indicator of the specific bacterial species. In this investigation, the absorption spectrum of the culture filtrate of *Streptomyces* sp. US7 MTCC 8723 displayed two major peaks, one at 345 nm and the other at 390 nm, as shown in Fig. 2. Similar observation has also been reported with *Streptomyces coelicolor*, which produces two antibiotics, a blue colored actinorhodin (ACT) and a red colored undecylprodigiosin (Ryu *et al.*, 2006). It has also reported that the actinomycetes often produce two or more antibiotics simultaneously having different spectral peaks (Krassilnikov, 1981). The first peak (345 nm) of *Streptomyces* sp. US7 MTCC 8723 with  $\lambda_{max}$  at 347 nm was most likely a flavone glycoside-like antifungal compound (Jensen *et al.*, 1998). The second peak was at 385 nm which is the  $\lambda_{max}$  of antibiotic LL-E 19085 beta and gamma (Carter *et al.*, 1992). It has also been observed that, in *Streptomyces* sp. US7 MTCC8723, the absorbance values of metabolites increased for up to 30 days of incubation and then decreased thereafter. This may be due to feedback inhibition by secondary metabolites (Zahn *et al.*, 2001).

Strains of *Streptomyces* sp. have the exceptional ability to produce a broad range of low molecular weight compounds of antibacterial and antifungal properties (Hodgson, 2000; Watve *et al.*, 2001; Fulgueira *et al.*, 2004). Environmental factors, such as carbon and nitrogen sources, oxygen tension, temperature, pH and method of cultivation can affect the timing and extent of production of antibiotics and other secondary metabolites (Martin and Demain, 1980; Iwai and Omura, 1982; Bhattacharyya *et al.*, 1998) as similar to our study. Moreover, in this study *Streptomyces* sp. US7 MTCC 8723 produced the maximum antibiotic at about neutral pH and at  $35 \pm 1^\circ\text{C}$ , consistent with previous findings. For example, *Streptomyces rochei* AK 39 also exhibited optimal results against dermatophytes when grown on

SCA medium with pH 7 and temperature  $37^\circ\text{C}$  (Augustine *et al.*, 2005).

The growth profile, closely coupled with the metabolic capacities of the producing organism, greatly influences the biosynthesis of antibiotics. For example, nutrient limitation in *Streptomyces* has been shown to trigger differentiation and induction of bacterial morphology and secondary metabolism (Braná and Demain, 1988). Also, it has been suggested by Augustine *et al.* (2005) that the maximum production of metabolites occurs in the late log phase and remains constant during the stationary phase. *Streptomyces* sp. US7 MTCC 8723 similarly showed an increase in biomass up to 21 days of incubation (late log phase). Maximum yield of antimicrobial metabolites was observed in the late log phase and it remained steady during the stationary phase (up to 30 days) indicating the direct relationship between growth rate and metabolite production.

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 continuing (James and Edwards, 1989). It has also been suggested that the protein secretion is invariably linked to the production of graniticin antibiotic in *Streptomyces thermoviolaceus*. *Streptomyces* sp. US7 MTCC 8723 showed continuous increase of protein in broth up to 60 days of incubation whereas decline in antibiotic production during 30 to 60 days of incubation.

#### 5. CONCLUSION

The newly isolated strain *Streptomyces* sp. US7 MTCC8723 showed broad antimicrobial spectra as it inhibited Gram-positive bacteria, Gram-negative bacteria as well as fungi. These significant results make the strain suitable for further investigation and industrial exploitation.

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