

Production of antibiotic from actinomycete from unexplored region of Kolli Hills, Tamilnadu and antibacterial activity against UTI pathogens

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INTRODUCTION

Actinobacteria are a source of a broad range of valuable and prominent pharmaceutically active metabolites [1]. More than half of the discovered secondary metabolites are attributed to actinobacteria [2]. Some of the major metabolites produced by actinomycetes include; antibiotics [3], antitumour agents [4] and enzymes [5]. Streptomycetes produce more than 70% of the available antibiotics [6] including; macrolides, tetracyclines and nucleoside [7]. The genus *Streptomyces* has been widely studied due to its ability to produce novel metabolites which have high commercial returns.

In a world faced with an increase in rise of drug resistant pathogens, and the level at which they are transmitted among people, there is a need to develop new and novel antimicrobial agents with novel mechanisms of action. Soil samples from unexplored areas are an undisputed source of actinomycetes that are a source of important antibiotics [8]. The objective in this study was to isolate, identify and characterize the actinomycete isolate, to optimize media for maximum yield production and to determine the antagonistic activity against UTI pathogens.

MATERIALS AND METHODS

Soil sample collection and isolation of actinomycete

Soil samples were collected from different sites (Rhizosphere and Grass land) of Kolli Hills, Namakkal District, TN, India using sterile spatulas. 1g of soil was suspended in 9ml of distilled water. 0.1ml of diluted aliquots- 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} were spread on plates containing starch-casein agar, HV agar and actinomycete isolation agar supplemented with Amoxicillin and cycloheximide to inhibit bacterial and fungal growth. The plates were kept at room temperature for 8days. Pure culture strains were preserved in 15% glycerol (v/v) at 4^oc [9].

Cultural and morphological characteristics

The actinomycete isolate was incubated on starch casein agar at 30^oc for 7days. The strain was carried out for gram staining, shape, size by phase contrast and SEM. Various biochemical tests were done to identify the isolate. The mycelium structure, colour and spore arrangement on mycelium were examined.

Test pathogens

The pathogens used include both gram positive and gram negative bacteria as well as fungi. They include, *Candida albicans* MTCC 227, *S. aureus* MTCC 3160, *E. coli* MTCC

1302, *Proteus* MTCC 1771, *P. aeruginosa* MTCC 1688 and *Klebsiella* MTCC 3384.

Primary screening of isolates for antimicrobial activity

Pure actinomycete isolates were centrally streaked on Mueller hinton agar plates and incubated at 30^oc for 6 days [10] Pathogens were streaked at right angles perpendicular to the actinomycete streak and incubated at 37^oc for 48h to evaluate antimicrobial activity [11].

Submerged fermentation

Seed culture media for submerged fermentation with the following composition (g/l) was used: Soluble Starch 10.0, Casein 0.3, glucose 10, glycerol 10, (NH₄)₂HPO₄1, (NH₄)₂SO₄3.5, CaCO₃5.10% of inoculums was added in 100 ml production media with composition: (g/l):sucrose 35, yeast extract 15.0, NaCl 4, KH₂PO₄3, K₂HPO₄2 and MnSO₄1. Inoculated cultures were grown in a rotary shaker at 200 rpm at 30^oC for 7 days [12].

Extraction and partial purification of metabolite

Solvent extraction method was used. Biomass was separated from growth medium by centrifugation at 8000 rpm for 9 min. A crude antimicrobial compound was extracted with equal volume of ethyl acetate. The brown colored residual active compound was then purified by using thin layer chromatography using methanol and chloroform as a running solvent system. Two fractions having different R_f values from the TLC plates were dissolved in 10% Dimethyl-sulfoxide. Purification of the crude compound was done by column chromatography technique on silica gel. chloroform- methanol (11:4) was used as a running solvent system [13].

Secondary screening

Antimicrobial activity of isolated active metabolite was studied by disc diffusion method. Freshly grown colonies of bacterial strains were inoculated into 25 ml of nutrient broth in a shaking water bath for 4–6 h until turbidity compared to that of the standard to 0.5McFarland. A sterile swab was dipped into inoculum and swabbed uniformly on the Mueller Hinton Agar surface. The inoculated plates were left at room temperature for 15 minutes to let any surface moisture to be absorbed before applying extract. 10 μ g/ml of the crude extract was impregnated on sterile antibiotic discs and placed on the surface of MHA medium. The discs were gently pressed on the medium. Tetracycline was used as a positive. The plates were incubated at 37^oC and the zones of inhibition were measured after 24 h. Methanol solvent served as a control [14].

Determination of Minimum Inhibitory Concentration

MIC was determined by broth two fold serial dilution using a gram positive and gram negative bacteria. 1ml of Nutrient Broth was dispensed in 10 tubes while 2 mL was dispensed in one test tube as a broth control. 1 mL of crude extract was added into first and second tubes while 2 mL of extract was added to test tube 12 as a crude extract control and serially diluted from tube 2 to 10. 1mL was discarded from tube 10 and added inoculums into tubes 1-10 and incubated at 37°C for 24 h after MIC value was determined by observing the bacterial growth [15].

Effect of temperature on growth

10mL of fresh seed cultures of the isolate inoculated in starch casein broth were added in 100mL conical flasks and incubated at different temperatures (20°C, 24°C, 28°C, 32°C, 36°C, 40°C) in a rotary shaker. After 72h, each broth was centrifuged at 10000rpm for 10 minutes and supernatant was assayed for antimicrobial activity [16].

Effect of pH

Starch casein broth was used to determine the effect of pH on the growth of the isolate. pH was adjusted as 5, 6, 7, 8 and 9 using HCL and KOH. The isolates were fermented after which they were tested for antimicrobial activity [17].

Effect of agitation

To study the effect of agitation on fermentation, 200 mL of starch casein broth was prepared in 5 conical flasks, sterilized and inoculated with seed inoculum. The flasks were kept for incubation on rotary incubator shaker at 50, 100, 150, 200 and 250 rpm for 7 days. Incubation temperature was 28°C. After 7 days fermented broth was centrifuged at 10,000 rpm at 4°C and supernatant was assayed for the antimicrobial activity [18].

Effect of fermentation time

200 mL Starch casein broth was prepared in 10 conical flasks, sterilized and inoculated with spore inoculum. The conical flasks were kept for incubation on rotary incubator shaker at 28°C and 150 rpm. One conical flask was removed daily; the fermentation medium was centrifuged at 10,000rpm for 9 minutes and assayed for antimicrobial activity by using Kirby Bauer disc technique. This procedure was repeated on 10 flasks for 10 days and observed for antimicrobial activity [13].

Effect of Inoculum volume

Effect of inoculum size was studied on antibiotic production. Spore suspension was inoculated as 2.5% in 50mL, 2.5% in 100mL, 2.5% in 150mL and 2.5% in 200mL of starch casein broth medium. The flasks were incubated at 28°C and 150 rpm on rotary incubator shaker for 1 week. After 7 days fermentation broth was centrifuged and supernatants were assayed for antimicrobial activity [19].

Ultraviolet and Fourier Transform Infrared spectra analysis

UV –spectra of the sample was subjected to comparison of general pattern and the maximum absorbance peaks and wavelength range. The sample was determined in the UV region (200-800) by using a HITACHI U-2900 spectrophotometer. FTIR spectra of sample was analyzed after homogenization of the sample with KBR.

The FTIR spectra were recorded on SHIMADZU AUX 220 spectrometer in the range of 4000-400 cm⁻¹ range and intensity was plotted against the wave number [20].

RESULT AND DISCUSSION

This study lays focus on isolation of potent antibiotic producing actinobacteria from rhizosphere and grassland of forest soil samples. 35 actinomycete strains were isolated from 5 soil samples sourced from different sites in Kolli Hills.

Out of the 35 isolates 20 isolates showed activity against at least one pathogen in primary screening. Of the isolated pure strains, one actinomycete culture, *Streptomyces sp.* ABK 07 was found as producing a wide spectrum of antimicrobial activity (Table 1).

Streptomyces sp. ABK07 utilized glucose, sucrose, maltose, mannitol while producing acid. It also utilized galactose and fructose but without acid production (Table 2).

Streptomyces commonly inhabit the soil and are saprophytes which have an ability to significantly produce antibiotics [21]. Selected isolate preliminarily subjected to phase contrast microscopy to observe the branched aerial hyphae arrangement. The characterization of *Streptomyces sp* is based on aerial mycelia the shape and ornamentation of spore surface. Physiological characters like temperature, utilization of sugar and reduction of nitrates. The brown vegetative mycelium and the aerial mycelium showed a light grey color. When examined under a scanning electron microscope, it showed features like flexuous sporophores arising from the aerial mycelium (Figure 1). Characteristics also shown by the *Streptomyces* genus [22].

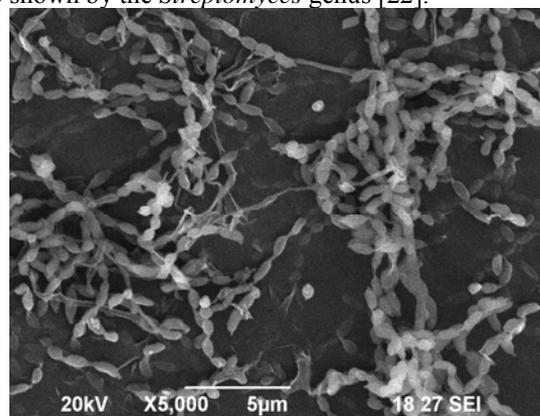


Fig 1: SEM analysis micrograph of *Streptomyces sp.* ABK 07.

The zone of inhibition in secondary screening ranged from 19-24 mm. Methanol was used as a control (Table 4). The chloroform extracted compound showed MIC values of 0.5µg/ml against *S. aureus*, 0.1µg/ml against *E. coli*, 3.0 µg/ml against *Proteus*, 1.0 µg/ml against *Klebsiella*, 0.5 µg/ml against *P. aeruginosa* and 0.2 µg/ml *Candida albicans*. This proved more effective compared to the compound extracted in ethyl acetate.

Actinomycetes fermentation is a complex process, which not only depends on the performance and fermentation medium, but also requires suitable factors which affect antibiotic production like inoculum volume, medium capacity, fermentation time, temperature, agitation rate and pH.

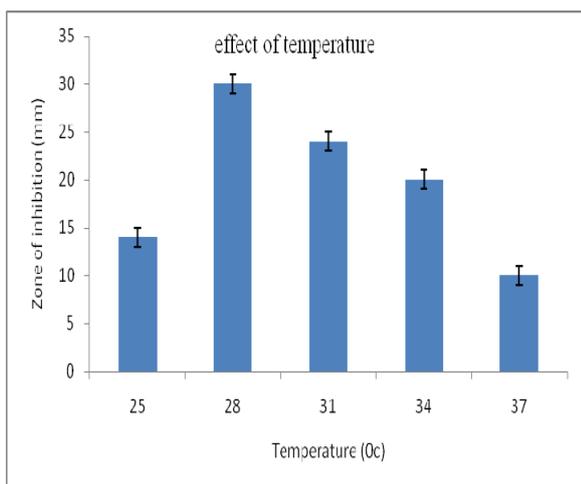


Fig 2: Effect of temperature on the growth of *Streptomyces* sp. ABK 07.

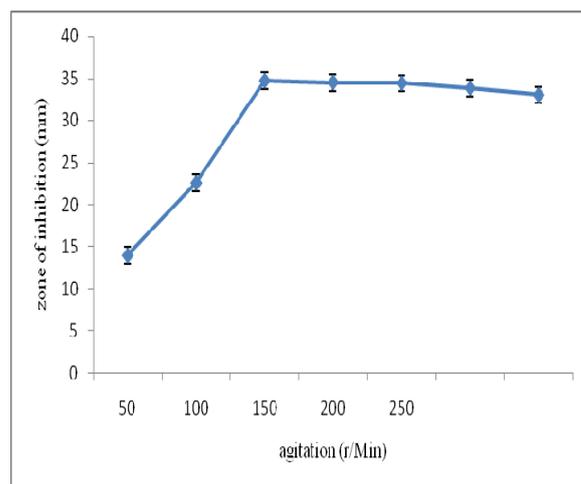


Fig 4: Effect of agitation rate on the growth of *Streptomyces* sp. ABK 07.

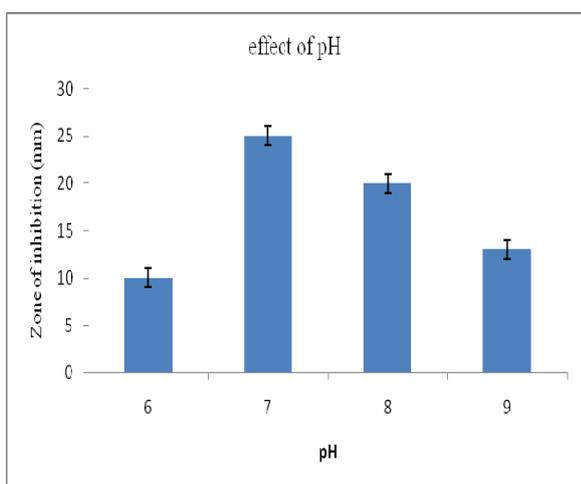


Fig 3: Effect of pH on the growth of *Streptomyces* sp. ABK 07.

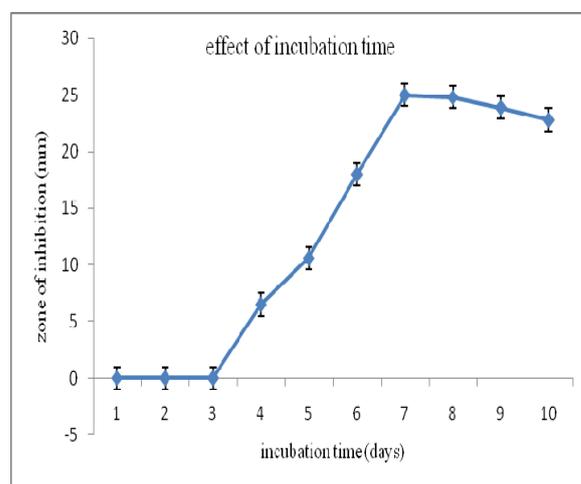


Fig 5: Effect of incubation time on the growth of *Streptomyces* sp. ABK 07.

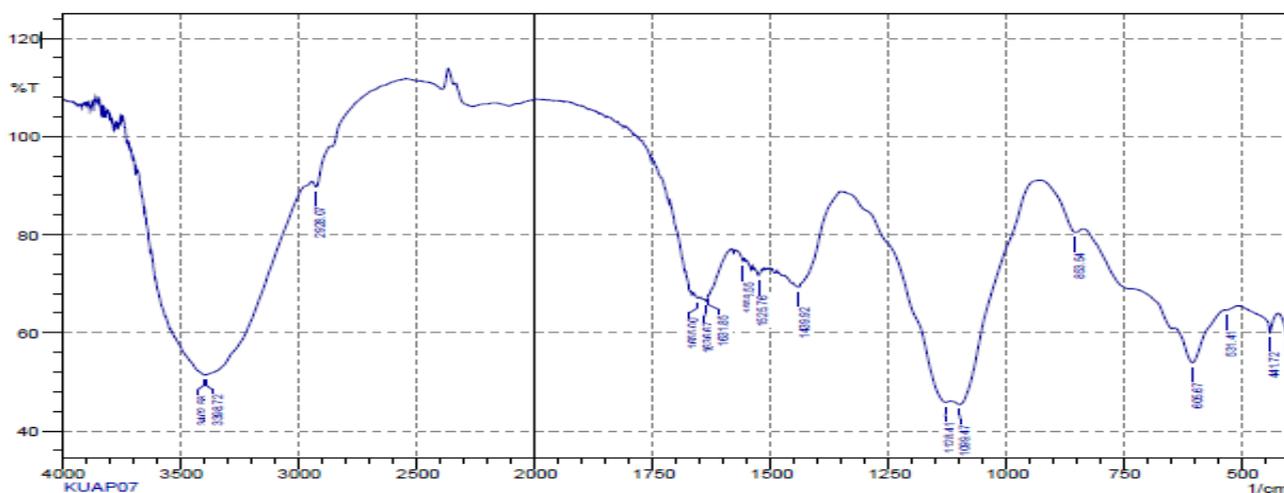


Fig 7: FTIR analysis of *Streptomyces* sp. ABK 07.

Zone of inhibition (mm)						
Pathogen Isolate	<i>C. albicans</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>Proteus</i>	<i>P. aeruginosa</i>	<i>Klebsiella</i>
ABK 01	-	07	10	09	05	-
ABK 02	-	-	06	-	-	-
ABK 03	15	12	13	14	12	13
ABK 04	-	07	10	-	06	-
ABK 05	03	02	08	-	06	-
ABK 06	06	13	11	12	10	09
ABK 07	12	14	15	16	17	16
ABK 08	11	10	11	11	07	08
ABK 09	-	-	-	-	05	-
ABK 10	01	02	05	03	06	-
ABK 11	07	14	10	12	14	10
ABK 12	-	-	-	07	-	02

Table 1: Zone of inhibition of pathogens.

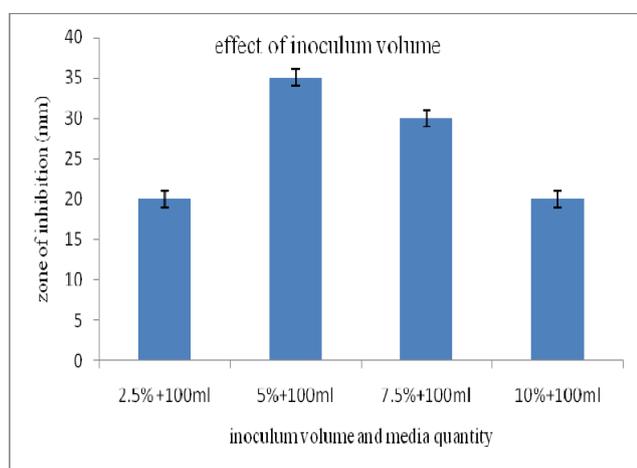


Fig 6: Effect of Inoculum volume on the growth of *Streptomyces sp. ABK 07*.

Sugar fermentation with acid production	
Glucose	++
Mannitol	++
Sucrose	++
Maltose	++
Sugar fermentation without acid production	
Xylose	--
Galactose	--
Fructose	--
Ammonia production	+ve
Catalase	+ve
Methyl red	-ve
Starch hydrolysis	+ve
Indole production	-ve
TSI	+ve

Table 2: Biochemical characteristics of *Streptomyces sp. ABK 07*

S.No	Pathogen	Methanol (zone in mm)	<i>Streptomyces sp. ABK07</i> (zone in mm)
1.	<i>C. albicans</i>	-	20
2.	<i>S. aureus</i>	-	19
3.	<i>E. coli</i>	-	24
4.	<i>Proteus</i>	-	21
5.	<i>P. aeruginosa</i>	-	18
6.	<i>Klebsiella</i>	-	23

Table 3: Zone of inhibition in secondary screening.

Streptomyces sp. ABK07 was cultivated at different temperatures ranging between 25°C and 37°C and produced an antimicrobial compound that inhibited the growth of *E. coli* between this temperature range. The maximum zone of inhibition rate reached 30mm at 28°C (figure 2). The inhibitory rate of the compound increased firstly and then decreased. This might be due to the effect of higher or lower temperature than optimum. Very low temperatures inhibit the production of antimicrobial compound. As temperature increased, there was an increase in metabolite production till the optimum temperature was reached after which increased temperatures could control the production. *S. warraensis* was reported secrete antibiotic at an optimum temperature of 30°C [23] while SUN-A2 secreted antibiotic at an optimum temperature of 28°C [24].

Initial pH of fermentation medium had a great impact on the antibacterial activity of *Streptomyces sp. ABK07*. The antibacterial activity increased with the increasing initial pH from 6.0 to 9.0, but any further increase in its values resulted in decreased antibacterial activity. This could have been due to too high or low initial pH which would decrease the production of active substance which affected the inhibitory effect. Under acidic conditions, it decreased significantly. At pH 7.0, the maximum zone of inhibition measured 25mm (figure 3). Some reports regarding the role of pH in production of bioactive compounds by microorganisms have been reported [24].

Rotary speed affects oxygen supply. The supply of oxygen for the fermentation process was provided by agitation and aeration. The inhibitory rate of *Streptomyces sp.* ABK07 initially increased. At agitation speed 150, the zone of inhibition measured was at 35mm (figure 4). Upon increasing the agitation rate, the inhibitory rate gradually decreased. *S. scelerotium* YJ1 was shown as having a maximum inhibitory rate at the agitation speed of 180r/min [25].

Time is a major factor affecting fermentation. Increasing time may not necessarily translate to increased secondary metabolites. This is because more toxins could be produced which could hinder the production of antimicrobial compounds [26]. Mycelia growth was observed between days 3-10 where day 7 exhibited maximum growth after which there was a slow decline (figure 5).

Inoculum volume affects metabolite accumulation. Low inoculums quantity may reduce product formation. Higher inoculums quantity may also hinder product formation due to accumulation of toxic substances and also may cause reduction of dissolved oxygen [27]. Maximum inhibition rate was achieved at 5% and 100ml (figure 6).

Antibiotic production is pre determined by supplementation of the growth medium with both carbon and nitrogen sources (Dermain 1989). *Streptomyces sp.* ABK 07 grew on all carbon supplemented media while producing the antibiotic but the maximum antibiotic production was realized in the media supplemented with 1.0% sucrose. The growth and antibiotic production reduces with either increase or decrease of concentration of sucrose. Sucrose in this study supports antibiotic production just like it supported A2D to produce bioactive metabolites [28]. The compound was confirmed as a secondary metabolite as its production started in the stationary phase.

Column chromatography was used for partial purification by using a solvent system of chloroform/methanol (1:4).

Two fractions with different R_f values were obtained. The fraction with R_f value 0.54 and UV_{max} at 320nm in chloroform exhibits antimicrobial activity against all the test UTI pathogens.

The fraction with R_f value 0.36 and UV_{max} at 220nm in methanol showed greater inhibition towards Gram positive pathogens as compared to Gram negative pathogens. The compound with R_f value 0.54 was characterized by FTIR. Infrared spectra exhibited absorption at 3402 and 3398 cm^{-1} , indicating a hydroxyl group and absorption at 1655 and 1631 cm^{-1} indicated a double bond of polygenic compound while absorption at 1665, 1636, 1631 cm^{-1} indicated a hydrocarbon chassis (figure 7). Similar results were recorded by Augustine *et al.*, [17] for their ethyl acetate extract of *S. albidoflavus* PU23 whose absorption at 3296 and 1031.8 cm^{-1} indicated hydroxyl groups and absorption at 1639 cm^{-1} indicated a double bonding.

CONCLUSION

The strain *Streptomyces sp.* ABK07 showed antimicrobial activity against UTI pathogens. The MIC values of the antimicrobial agent against the test pathogen indicated a

potent activity and as such could be used in the generation of new antimicrobial agents. The findings of this study illustrate that naturally occurring forest actinomycetes have a great potential in the production of antimicrobial agents which could aid in discovery of new antibiotics. This could be of great importance especially in their use to modify urinary catheters to prevent the spread of nosocomial urinary tract infections.

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