

Studies on the antibacterial activity of the Actinomycetes isolated from the Khumbu Region of Nepal.

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Abstract

Antibacterial activity of actinomycetes isolated from Lobuche area (5000-5300 meter in height) and Lukla area (2660 meter in height) in Khumbu region has been studied. A total of 106 actinomycetes were subjected to primary screening by perpendicular streak method against Gram-positive (Bacillus subtilis and Staphylococcus aureus) and Gram-negative (Enterobacter aerogens, Escherichia coli, Klebsiella species, Proteus species, Pseudomonas species, Salmonella typhi and Shigella species) test bacteria. It was observed that 2 isolates were active against only Gram-negative bacteria, 8 against Gram-positive and 26 against both Gram-positive and Gram-negative bacteria.

Altogether 36 putative isolates were subjected to secondary screening by agar well method to further test the capabilities of primarily screened organisms. Selected isolates (20) from the secondary screening belonged to the genera Streptomyces (10), Streptovercillium (4), Saccharopolyspora (3), Micromonospora (2) and Actinosynema (1).

Finally 2 isolates (Streptomyces species and Saccharopolyspora species) were selected for further study on the basis of (a) broad spectrum activity and (b) larger zone of inhibition in comparison to others. The antibacterial substances were extracted with ethyl acetate from isolate-inoculated starch-casein broth fermented for 7 days at 28°C by solvent extraction method. Minimum bactericidal concentration (MBC) of ethyl acetate extract against Staphylococcus aureus were 1.25 mg/ml for Saccharopolyspora species and 5mg/ml for Streptomyces species. Thin layer chromatography (TLC) of the ethyl acetate extracts were carried out in duplicate using Chloroform: methanol (4:1) as solvent system and Tetracycline as reference antibiotic. Under UV light they gave greenish yellow spots with R_f value 0.88 for the antimicrobial from Streptomyces species and 0.90 for that from Saccharopolyspora species. In bioautography (using Staphylococcus aureus as test organism) inhibition zones were obtained and they were associated with the yellowish green spots of the chromatogram as detected under UV light. This may indicate the same compounds were responsible for the antibacterial activity of those actinomycetes isolates.

Introduction

The actinomycetes are Gram positive bacteria having high G+C (>55%) content in their DNA. The name 'Actinomycetes' was derived from Greek 'aktis' (a ray) and 'mykes' (fungus) and given to these organisms from initial observation of their morphology. Actinomycetes were originally considered to be an intermediate group between bacteria and fungi but now are recognized as prokaryotic organisms.

The majority of actinomycetes are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants. Actinomycetes population has been identified as one of the major group of soil population (Kuster 1968), which may vary with the soil type.

The actinomycetes are noteworthy as antibiotic producers, making three quarters of all known products; the *Streptomyces* are especially prolific and can produce a great many antibiotics and other class of biologically active secondary metabolites. They cover around 80% of total antibiotic product, with other genera trailing numerically; *Micromonospora* is the runner up with less than one-tenth as many as *Streptomyces*. If we include secondary metabolites with biological activities other than antimicrobial, actinomycetes are still out in front, over 60%; *Streptomyces* spp. accounting for 80% of these (Hopwood, *et al.*, 2000).

Due to large geographic variation, there is large variation in soil type and their contents in Nepal and hence it is quite likely that the distribution of antibiotic producing actinomycetes is also variable. This study is carried out to screen the antibiotic producing actinomycetes from higher altitude, Khumbu region, of Nepal which is situated at the lower part of Mount Everest base camp.

Materials and methods

The specimens (actinomycetes) used in this study were isolated from the soils of Khumbu region. Soils from different places of Khumbu region were brought to the laboratory in aseptic condition. Actinomycetes from the soil had been isolated by pour plate technique on Starch-casein agar and Glycerol-arginine agar after serial dilution in distilled water. Dry colonies of actinomycetes were selected and isolated. Thus isolated colonies had been preserved in Glycerol based media and stored at -20°C. The actinomycetes of which antimicrobial activity should be determined were revived by streaking on Starch-Casein agar and incubated at 28°C for 7 days.

Screening of actinomycetes for antimicrobial activity: The screening method consists of two steps; Primary screening and secondary screening.

In primary screening the antimicrobial activity of pure isolates were determined by perpendicular streak method (Egorov, 1985) on Nutrient agar (NA). The test organisms used were; *Bacillus subtilis*, *Staphylococcus aureus*, *Enterobacter aerogens*, *Escherichia coli*, *Klebsiella* species, *Proteus* species, *Pseudomonas* species, *Salmonella typhi* and *Shigella* species.

Secondary screening was performed by agar well method against the standard test organisms *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Bacillus subtilis*, and *Proteus* spp.

Characterization of actinomycetes: The potent actinomycetes selected from secondary screening were characterized by morphological and biochemical methods. Morphological methods consist of macroscopic and microscopic methods. The microscopic characterization was done by cover slip culture method (Kawato and Sinobu, 1979). The mycelium structure, color and arrangement of conidiospore and arthrospore on the mycelium was observed through the oil immersion (1000X). The observed structure was compared with Bergey's manual of Determinative Bacteriology, Ninth edition (2000) and the organism was identified. Various biochemical tests performed for the identification of the potent isolates are as follows: Casein hydrolysis, Starch hydrolysis, Tween 20 hydrolysis, Urea hydrolysis, Esculin hydrolysis, Acid production from sugar, NaCl resistance, Temperature tolerance.

Fermentation process: Fermentation was carried out in a 1L Erlenmeyer flask following the procedure as described by Liu *et.al* (1992).

Isolation of antibacterial metabolites: Antibacterial compound was recovered from the filtrate by solvent extraction method following the process described by Westley *et.al*, 1979. and Liu *et.al*, 1986. Ethyl acetate was added to the filtrate in the ratio of 1:1(v/v) and shaken vigorously for 1 hour for complete extraction. The ethyl acetate phase that contains antibiotic was separated from the aqueous phase. It was evaporated to dryness in water bath at 80°-90°C and the residue obtained was weighed. Thus obtained compound was used to determine antimicrobial activity, minimum inhibitory concentration and to perform bioautography.

Determination of the antimicrobial activity: The antimicrobial activity was determined by agar well method (Sen. *et al.*, 1995). The partially purified extract obtained by the evaporation of the ethyl acetate extract was dissolved in 1 ml 0.2M phosphate buffer (pH 7.0). Then 100µl of it was loaded into well bored and test organism (0.5 McFarland turbidity standard) swabbed Muller Hinton agar plates. The plates were incubated at 37°C for 18-24 hrs and examined. The diameter of the zones of complete inhibition was measured to the nearest whole millimeter.

Determination of minimum inhibitory concentration: It was determined by the serial dilution of the antimicrobial in nutrient broth, two fold dilution at each time, against *Staphylococcus aureus*.

Thin layer chromatography and Bioautography: Silica gel plates, 10X20 cm, 1mm thick, were prepared. They were activated at 150°C for half an hour. Ten microliters of the ethyl acetate fractions and reference antibiotics were applied on the plates and the chromatogram was developed using chloroform: methanol (4:1) as solvent system. The plates were run in duplicate; one set was used as the reference chromatogram and the other was used for bioautography. The spots in the chromatogram were visualized in the iodine vapour chamber and UV chamber.

Muller Hinton agar inoculated with *Staphylococcus aureus* was poured over the chromatogram and the plate was incubated overnight at 37°C in sterile condition. The next day the inhibition zones were noted and the R_f values of the antimicrobials were determined.

Results

Out of 106 actinomycetes subjected for primary screening process, only 36 isolates showed the activity against test organisms. Of the 36 isolates, 2 were active against only gram negative organism, 8 against gram positive organisms and 26 against both gram positive and gram negative organisms. Among them, 31 of the isolates were active against *Bacillus subtilis*, 27 against *Staphylococcus aureus*, 17 against *Escherichia coli*, 15 against *Salmonella typhi* and 14 against *Proteus* species.

Out of the 36 isolates that were subjected for the secondary screening, 23 isolates were active against *Bacillus subtilis*, 23 against *Staph. aureus*, 17 against *E. coli*, 10 against *Proteus* species and 6 against *Salmonella typhi*.

The results can be represented in the bar diagram as follows:

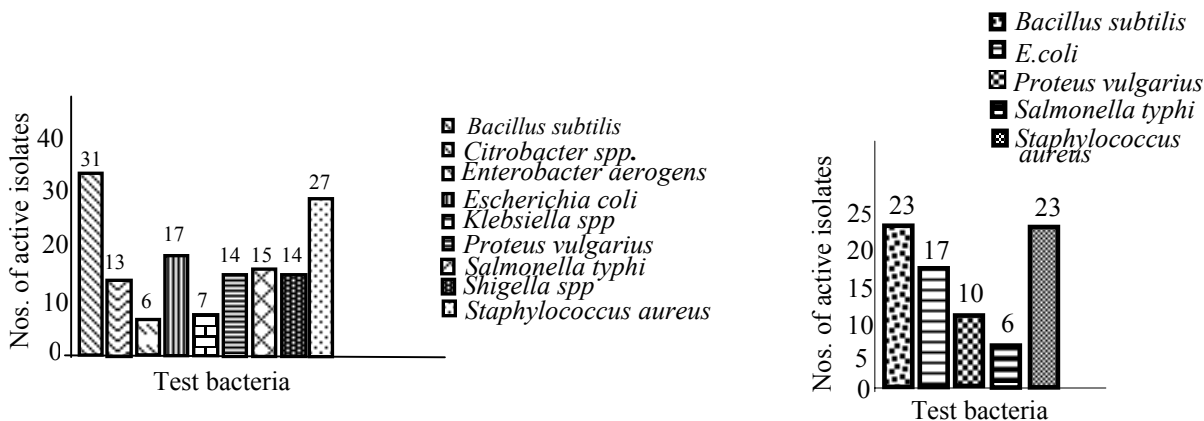


Fig 1: Activity shown by actinomycetes in primary screening.

Fig2: Activity shown to test bacteria in secondary screening.

Identification: The identification of the potent antibiotic producing strains reveals that most of the specimens belong to the genus *Streptomyces* (10) followed by *Streptoverticillium*(4), *Saccharopolyspora*(3), *Micromonospora* (2) and *Actinosynema* (1).

Two potent isolates were selected for fermentation on the basis of their broad spectrum of activity and largest zone of inhibition. They were found belonging to the genera *Streptomyces* and *Saccharopolyspora*.

Minimum inhibitory concentration: The minimum inhibitory concentration for the extract from *Streptomyces* spp was 5mg/ml and that from *Saccharopolyspora* spp was 1.25mg/ml.

Thin layer chromatography and Bioautography: The spot given by the extract of *Streptomyces* spp was a circular with R_f value 0.88 and that of *Saccharopolyspora* spp was an extended spot with R_f value 0.90. The fluorescence colours of the spots were greenish yellow. The reference antibiotic, Tetracycline, didn't move with the solvent system.

In bioautography the *Streptomyces* spp gave an inhibition zone of 20mm diameter and the *Saccharopolyspora* spp gave inhibition zone of size 10x80mm. The reference antibiotic Tetracycline gave the inhibition zone of 25mm diameter at the origin.

Discussion

The putative isolates of primary screening when subjected to secondary screening, showed different activity from that of primary screening; some of the active isolates didn't show the activity in the secondary screening while some showed little activity and some showed improved activity. According to Bushell (1993), during the screening of the novel secondary metabolite, actinomycetes isolates are often encountered which show antibiotic activity on agar but not in liquid culture.

The result of primary and secondary screening reveals that most of the active isolates were active against gram positive bacteria (*Bacillus subtilis* and *Staph. aureus*) than gram negative bacteria. The reason for different sensitivity between gram positive and gram negative bacteria could be ascribed to the morphological differences between these microorganisms, gram negative bacteria having an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, The gram positive should more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier (Scherrer & Gerhardt, 1971). Although various biochemical tests were performed, it was unable to identify the actinomycetes up to species level due to the lack of other tests. According to Kutzner (1972) for proper identification of genera and species of actinomycetes, besides morphological and physiological properties, various other biochemical properties such as cell wall chemo type, whole-cell sugar pattern, peptidoglycan type, phospholipids type and G+C% of DNA should be determined.

The minimum inhibitory concentration (MIC) for the antimicrobial extracted from *Streptomyces* spp was 5mg/ml and that from *Saccharopolysporaso* spp was 1.25mg/ml. This shows that the antimicrobial from *Saccharopolyspora* spp was more active than from *Streptomyces* spp but there are various factors affecting the activity. The *Streptomyces* spp can be a poor fermenter than the later one or the solvent used for extraction may not be suitable for it or the compound may not be properly extracted by the solvent.

The MIC is not a constant for a given agent, because it is affected by the nature of the test organism used, the inoculum size, and the composition of the culture medium, the incubation time, and aeration.

For complete characterization of an antibiotic it should be isolated in pure form as a single component but this is impractical in a screening programme like this. However, a little effort was made in this approach. According to the TLC separation, the two extracts yielded components with R_f values similar to the antibacterial compounds as visible on bioautogram. In addition, the inhibition zones were associated with yellowish green spots which had been detected under UV radiation. This may mean that the same compounds are responsible for antibacterial activity of those isolates.

Although the antimicrobial agents obtained in this study can't be declared as new antibiotics, there is the probability of finding new antibiotics in Nepal because of its wide biodiversity. For proper identification of the antimicrobial extracts it is necessary to obtain in pure form, which requires a series of purification process and different chemical analysis such as HPLC, Spectroscopy and other sophisticated techniques. As we know the land of Nepal is virgin in this field, so lots of works should be done to explore the new antibiotics because any new antibiotics and its producing organism have been a great demand.

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