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Records of potential antimicrobial activity of soil actinomycetes isolated from a community forest in Ban Khoklam Sang Aram, Udon Thani Province

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Abstract: The WHO (2015) released a report of an increasing number of infections due to the global antimicrobial drug resistance crisis. Unexplored areas may be possible for sources of actinomycetes with potent antimicrobial compounds. This study was directed towards isolation of actinomycetes that produce antimicrobial substances from unexplored soil in a community forest in Ban Khoklam Sang Aram, Udon Thani Province. The survey showed diversity of actinomycetes and rare actinomycetes in this area. Ten of 232 isolated actinomycetes showed potent antimicrobial activity by growth inhibition of *Bacillus subtillis*, *Candida albicans*, *Escherichia coli*, *Pseudomonas aeroginosa* and *Staphylococcus* spp. after testing using agar disc diffusion and agar well diffusion tests. These isolates were identified as species in the genera *Streptomyces* (9 isolates) and *Microbispora* (1 isolate).

Keywords: Microbial diversity, actinomycetes, Antimicrobial activity, Soil microorganism, Pathogenic microorganism

Introduction

Actinomycetes are Gram-positive bacteria. They are unique in their formation of branching filaments with fungi like hyphae and asexual spores. Their bacterial DNA has a high G+C content, 55-75% (Lechevalier and Lechevalier, 1967; Embley and Stackebrandt, 1994). Actinomycetes is a major microbial population that is widely distributed and inhabits the soil (Jaralla et al., 2014). They play role in soil biodegradation and humus formation (McCathy and Williams, 1992; Agadagba, 2014). Moreover, many actinomycetes are pharmaceutically important for production of secondary metabolites as antibiotics, vitamins and enzymes (Shahidi et al., 2004; Xu et al., 2005; Arifuzzaman et al., 2010). Soil actinomycetes produce many of currently used antibiotics including Erythromycin, Gentamycin, Rifamycin and Streptomycin (Jeffrey, 2008). Therefore actinomycetes are potentially important strains for producing new bioactive compounds, especially antibiotics that have diverse clinical effects and have activity against many pathogens (Atta and Ahamd, 2009; Usha et al., 2011).

This work was aimed to investigate diversity of an actinomycetes population in the soil of a community forest in Ban Khoklam Sang Aram, Udon Thani Province and their antimicrobial activities, for the first time. This forest has not yet been studied. Additionally, this area yields *Streptomyces* species and rare actinomycetes strains with high antimicrobial activity.

Materials and Methods

Collection of soil samples

Seven soil samples were randomly collected from various sites in a community forest in Ban Khoklam Sang Aram, Udon Thani Province. The seventh sites where the collected soil sample were takes were diverse. They include 1) loam with a leaf covering, 2) loam with a decayed wood covering, 3) fine sand with no vegetation and a leaf covering that served as drainage for rainfall and 4) sandy loam surrounding plant roots. The soil was taken at a depth of 1-10 cm from the soil surface. Approximately 500 grams of each soil sample was collected and was placed in sterile polyethylene bags and kept at room temperature. These were promptly transferred to a laboratory for analysis of their actinomycetes population.

Preparation of soil samples

The soil samples were air-dried at room temperature until a constant weight was attained and were stored at 4°C prior to processing for actinomycetes isolation. They were pretreated to stimulate actinomycetes growth and eliminate unwanted Gram-negative bacteria. Each soil sample divided into two parts for separate pretreatment methods. One part was pretreated with wet heat and the other was pretreated with air dry plus calcium carbonate ($CaCO_3$) following the methodology of Fang et al. (2017). Then the samples were further examined for diversity of actinomycetes colonies and actinomycetes isolates.

Enumeration isolation and purification of actinomycetes

Actinomycetes in each soil were isolated using a 10-fold serial dilution of soil and spread plate technique. Diluted soil solution was spread on a humic acid (HA) medium (Hayakawa and Nonomura, 1987) and starch-casein agar (SCA) (Okazaki et al., 1983) supplement with 1 mg/mL of nalidixic acid and 1 mg/mL of cycloheximide. All spread plates were incubated at 37°C for a period of 14 days.

A number of diverse morphological colonies appeared in each dilution on both the HA and SCA media on day 14. The various colony morphologies were carefully counted and recorded. Then, the density of the actinomycetes population was determined in units of CFU/g dried soil as the number colonies on a plate times the dilution factor.

Various colonies on HA and SCA media were then picked with sterile needles and transferred to an International Streptomyces Project 2 (ISP2) medium. Each isolate was purified using a streak plate technique and cultured at 37°C for a period of 3-5 days until pure single colonies appeared.

Preliminary Screening for antimicrobial activity

Preliminary screening of the antimicrobial activity of purified isolates was done using a streak plate technique following Lemons et al. (1985). One isolate was long single streak at the corner of ISP2 solid medium plate. Four pathogenic strains were used to investigate the antagonistic activity of actinomycetes isolates. They were *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. These pathogenic microorganisms were suspended (0.5 McFarland) and streaked perpendicular to the actinomycetes strain using a sterilized loop. The plate was then incubated at 37°C for a period of 14 days. Clear zones, indicating microbial inhibition, and their widths were determined in millimeters.

Secondary screening for antimicrobial activity

Each purified isolate expressing growth inhibition in the preliminary screening was cultured in 100 ml ISP2 broth on a 250 ml rotary shaker (180 revolutions per minute, rpm) at 28±2°C for 4 days. They were then transferred to a new *YEME* broth supplemented with 0.1% $CaCO_3$, as a production medium. The cultures were incubated a rotary shaker (180 rpm) at room temperature for 14 days. The culture broth was filtered through Whatman No.1 filter paper to separate the mycelium. A clear supernatants were obtained that were extracted three times with ethyl acetate at a 1:1 (v/v) ratio with vigorous shaking for 1 hour according the method of Rao et al. (2017). The bioactive compounds were in the ethyl acetate phase that separated from the aqueous phase. The ethyl acetate phase was evaporated under a reduced vacuum at 80°C. The obtained bioactive crude extract was used to test the antimicrobial activity of isolates using agar disc diffusion and agar well diffusion tests.

Secondary screening for antimicrobial activity

The antimicrobial activity of purified isolates expressing growth inhibition in the preliminary screening were confirmed by agar disc diffusion and agar well diffusion methods as described by Mohseni et al. (2013) and Omar et al. (2000), respectively. Nystatin and nalidixic acid agents were used as controls for anti-Gram positive and anti-Gram negative bacterial activity, respectively.

Morphological identification of actinomycetes

Preliminary identification of actinomycetes isolates grouped the bacterial as *Streptomyces* and non- *Streptomyces*, (rare actinomycetes). It was performed by observing the growth rates of bacteria on an International Streptomyces Project (ISP) medium (Shirling and Gottli, 1966). *Streptomyces* grew faster than non- *Streptomyces*, as was also reported by Abdullah et al. (2016).

Isolates were identified at the genus level based on microscopic and macroscopic studies of actinomycetes following Shirling and Gottileb (1966), Muthu et al. (2013) and Barka et al. (2015). Macroscopic examination considered colony features such as size, shape, color, absence or presence of aerial mycelium and spore formation. Microscopic characteristics observed were aerial and substrate mycelium, conidia, spore type, and especially spore chains.

Result & Discussion

Enumeration and isolation of actinomycetes

Actinomycetes in soil samples were isolated using HA medium and SCA medium supplement with nalidixic acid (as antifungal agent) and cycloheximide (as antibacterial agent) at 37°C for 7-14 days. The diversity of the actinomycetes population was viewed from various types of colony morphology, size, color, elevation, surface and pigmentation, as they grew on a solid medium.

The results indicated a high diversity of actinomycetes from soil samples collected from a community forest in Ban Khoklam Sang Aram, Udon Thani Province, on both media. This data is shown in Figure 1. The various soil samples had actinomycetes populatons in the range of 1.1×10^2 - 5.3×10^2 CFU/g dried soil on HA plates and 2.1×10^2 - 9.4×10^2 CFU/g dried soil on SCA plates. The various colonies had obviously different colony characteristic on the media. The highest numbers of colonies with different morphology were found on the SCA medium as it supported significantly more growth than the HA medium. Most actinomycetes isolates grew

better when hydrolyzing starch and casein than the humid acid in the HA medium (Ravel et al., 2000). The types of colonies grown on SCA medium are shown in Figure 2.

The most populated sample had 9.4 x 10^2 CFU/g dried soil on SCA medium, from the fourth site in the forest. Its soil was a fine sand without vegetation and the soil was saturated with water. This may have been due to a recent rainfall. Furthermore, the soil might contain soil nutrients, such as organic carbon, that originated from other sites. According to Stackebrandt et al. (1991) and Baby et al. (2002), fertile soils or soil containing organic carbon, nitrogen, and phosphorus is an important soil feature that can enhance the grow the rate and diversity of an actinomycetes population. Alternatively, the highest number of diverse colonies on HA medium was 5.3 x 10^2 CFU/g dried soil from soil at the seventh site. This soil was a loam with a decayed wood covering. The colony morphology from soil taken at this site was different than from other soil sources. So, it was of interest to investigate these bacteria. Actinomycetes isolates have been found on decomposing wood since some actinomycetes can produce hydrolytic enzymes to break down the lignocellulose of wood (Bontemps et al., 2013; Ding et al., 2004). The lowest number of colonies on both media was 2.1 x 10^2 CFU/g dried soil on SCA medium from soil at third site, as fine sand without vegetation. It was in an area where there was water flow due to rainwater runoff.

A total of 232 actinomycetes were isolated from seven soil samples collected at various locations within this community forest.

Primary screening for antimicrobial activity

All actinomycetes isolates underwent a preliminary screening for antimicrobial activity using an agar cross streak method. It found that ten isolates possess inhibitory activity against pathogenic fungi and bacteria. These were the 8.2.4HV, 8.2.5HV, 6.1HV-1, 20.2.6, 15.1, RH13-26, 11.1.2, 10. 4.1, 10.5, and RH12-1 isolates.

Secondary screening for antimicrobial activity

The ten isolates were further tested to confirm their antimicrobial activity using a crude ethyl acetate extract with disc diffusion and well diffusion methods. Their antimicrobial activity was measured in terms of a clear zone that formed. As shown in Table 1, many isolates were active against more than one of the tested pathogenic microorganisms. *Bacillus subtillis* growth was inhibited by eight isolates. These were 6.1HV-1, 20.2.6, 15.1, 11.1.2, 10.5, 8.2.4HV, 10.4.1 and RH12-1, ranked from highest to lowest antimicrobial activity. Maximum and minimum inhibition zones of *B. subtillis* of these isolates were 10 and 1 mm, respectively. *Staphylococcus aureus* growth was inhibited by seven isolates viz., 15.1, 11.1.2, 10.5, 8.2.4HV, 8.2.5HV, 10.4.1, and RH12-1. The isolates displayed maximum and minimum inhibition zones for *S. aureus* of 8 and 4 mm, respectively. *Pseudomonas aeruginosa* growth was inhibited by eight isolates *viz.*, 8.2.4HV, RH13-26, 15.1, 10.5, 8.2.5HV, 11.1.2, 10.4.1, and 20.2.6. The isolates displayed maximum and minimum inhibition zones for *P. aeruginosa* inhibition of 20 and 4 mm, respectively. *Candida albicans* growth was inhibited by three isolates viz., 8.2.4HV, 10.5, and 8.2.5HV. The isolates displayed maximum and minimum inhibition zones for *C. albicans* of 10 and 6 mm, respectively. No isolate showed growth inhibition of *E. coli*. Isolates 8.2.4HV and 10.5 showed inhibition against four pathogenic microorganisms, but not against *E. coli*. Some isolates (10.4.1 and RH12-1) showed no inhibition of pathogenic microorganisms. Actinomycetes isolates all produced secondary metabolites, such as bioactive antibiotics, to inhibit growth of some pathogens. These results are similar to those of Thangapandian et al. (2007) and Gebreyohannes et al. (2013) who studied antimicrobial actinomycetes isolated from rhizosphere soil, water, and sediment.

The isolate 10.5 displayed antimicrobial activity against four pathogens including *Bacillus subtilis*, *S. aureus*, *P. aeruginosa*, and *C. albicans*, comparing with other isolates. The results showed in Figure 3.

Identification of actinomycetes

A total of 232 microorganisms were preliminary identified as *Streptomyces* and non- *Streptomyces* (rare actinomycetes), by observing their growth rate on ISP medium. It was found that most of the isolates (206) showed the typical morphology of *Streptomyces* and few isolates (26) were found to be rare actinomycetes.

Only ten isolates expressed antimicrobial activity. They were identified at the genus level by examining their morphological characteristics, i.e., presence of aerial mycelium, conidia, pigment per Barka et al. (2015) and Shirling and Gottileb (1966). The results indicate that these isolates can be divided into two genera, *Streptomyces* and *Microbispore*. Isolates 8.2.4HV, 8.2.5HV, 6.1HV-1, 20.2.6, 15.1, 11.1.2, 10.5, RH13-26, and RH12-1 are *Streptomyces*. They have diverse types of spores including retinaculiapert, oligosporous, spira and spiral spores. Only isolate, 10.4.1, was a *Microbispore* spp. and it showed disporus spores as depicted in Figure 4.

Conclusion

This work clearly showed that soil samples collected from a community forest in Ban Khoklam Sang Aram, Udon Thani Province contain actinomycetes that possess antibacterial and antifungal activities. The data demonstrates the diversity of actinomycetes population in the form of colony morphology. Additionally, these isolates were effective in producing antimicrobial metabolites to against *B. subtilis*, *S. aureus*, *P. aeruginosa* and *C. albicans*. It is revealed that this location is a good source of antimicrobial actinomycetes, both *Streptomyces*

and rare-actinomycetes. Further work will be performed to determine their potential application to control human and plant pathogens.

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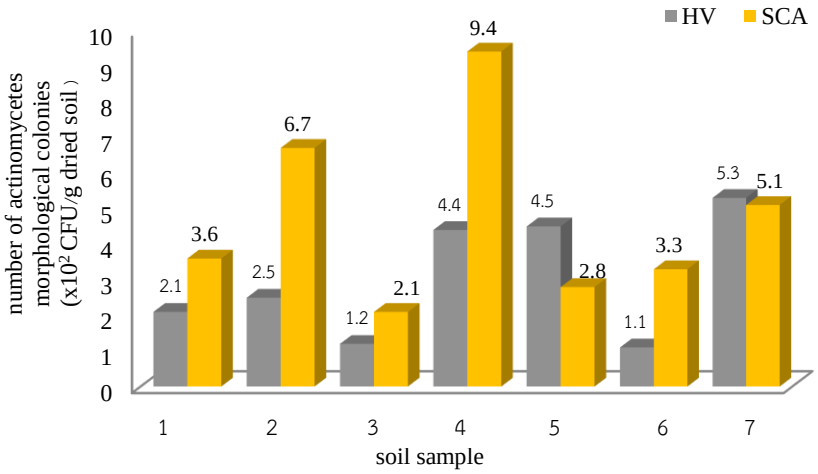

Figure 1. Morphological diversity of actinomycetes colony in soil samples collected from a community forest in Ban Khoklam Sang Aram, Udon Thani Province using HA and SCA media.

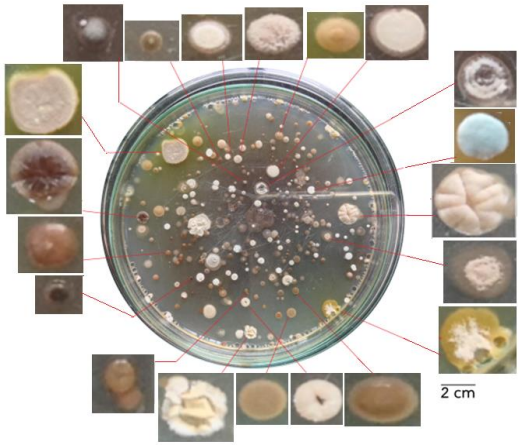

Figure 2. Various morphological colonies of actinomycetes growing on SCA medium supplemented with nalidixic acid and cycloheximide of culture broth of actinomycetes isolates.

Table 1. Antimicrobial activity of actinomycetes isolates showed inhibition zone against the tested pathogenic microorganisms.

Isolate	Inhibition (mm)				
	B. subtilis	***S. aureus***	***P. aeruginosa***	***E.coli***	***C. albicans***
8.2.4HV	1	4	20	0	10
8.2.5HV	0	4	7	0	6
6.1HV-1	10	0	0	0	0
20.2.6	10	0	4	0	0
15.1	10	8	8	0	0
RH13-26	0	0	10	0	0
11.1.2	10	8	7	0	0
10.4.1	3	4	4	0	0
10.5	5	8	8	0	9
RH12-1	3	3	0	0	0

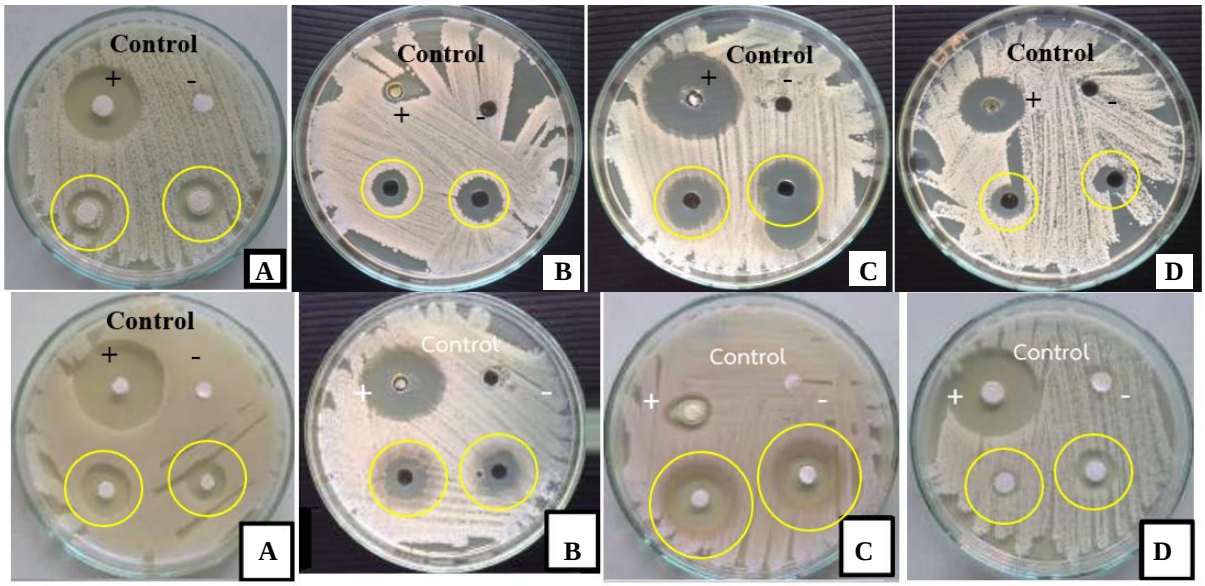

Figure 3. Antimicrobial activity of the 10.5 isolate against pathogens: A, *Bacillus subtilis*; B, *S. aureus*; C, *P. aeruginosa*; D, *C. albicans* using an agar disc diffusion method and an agar well diffusion method; nystatin as control for anti-gram positive bacteria; nalidixic acid as anti-Gram negative bacteria.

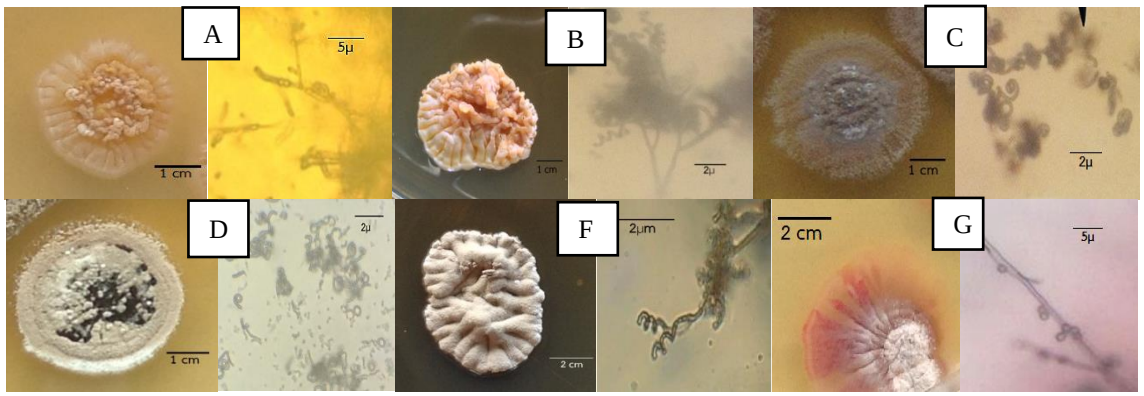

Figure 4. Morphology of colonies and spores of actinomycetes isolates: A, 8.2.4HV, retinaculiapert spore; B, 8.2.5HV, spirals spore; C, 6.1HV-1, spira spore; D, 15.1, oligosporous spore; F, RH12-1, spirals spore; G, 10.4.1, disporus spore.