**KMITL Sci. Tech. J. Vol. 15 No. 2 Jul. - Dec. 2015**

# Antimicrobial Activity of Acidophilic Actinomycetes Isolated from Acidic Soil

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## Abstract

Acidophilic actinomycetes were isolated from acidic soil samples on Starch Casein Agar, Soil Extract Agar and Humic Vitamin Agar, pH 4.5, containing nystatin and nalidixic acid. The total acidophilic actinomycete counts ranged from 3.9-8.2 x $10^3$ CFU/g of soil sample. Antibacterial activities of isolated actinomycetes were primarily screened by agar plug method. Three isolates exhibited antibacterial activity against all 4 tested bacteria, which were *Bacillus cereus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus.* Furthermore, 10 isolates showed antifungal activity against *Fusarium* sp., *Curvularia* sp. and *Colletotrichum gloeosporioides*. The 14-day YM culture broth and mycelium of isolate AA01 and AA02 were extracted with hexane, ethyl acetate and methanol. The crude extracts of AA01 and AA02 were then tested for antibacterial and antifungal activity, respectively. The MIC values of methanolic crude extract from AA01 against *B. cereus*, *P. aeruginosa*, *E. coli* and *S. aureus* were 5, 20, 40 and 5 mg/ml, respectively. The methanolic crude extract from AA02 at concentration of 30 mg/ml can inhibit at least 50% of the fungal growth.

**Keywords:** acidophilic actinomycete, antibacterial activity, antifungal activity, acidic soil.

## 1. Introduction

Soil microorganisms provide an excellent resource for the isolation and identification of therapeutically important products. Among them, actinomycetes are an important group producing antibiotics of agricultural and medicinal importance [1]. Actinomycetes are filamentous Gram-positive bacteria with true aerial hyphae, belonging to the phylum *Actinobacteria* (order *Actinomycetales*) that represents one of the largest taxonomic units among the 18 major lineages currently recognized within the domain Bacteria [2]. Actinomycetes population has been identified as one of the major groups in the soil population, which may vary with the soil type. They are excellent elaborators of biotechnological products such as antibiotics, industrial enzymes and other bioactive compounds. The secondary metabolites obtained from the class actinobacteria are of special interest because of their diverse biological activities such as antibacterial, antifungal, antioxidant, antitumor and antiviral. Around 23,000 bioactive secondary metabolites formed by microorganisms have been reported and over 10,000 of these compounds are produced by actinomycetes [3]. Acidophilic actinobacteria are aerobic and chemoorganotrophic organisms that grow in acidic environments [4-5]. They are potential sources of antimicrobial compounds [6] and

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acid-stable enzymes [7]. Their optimal growth occurs at a pH of approximately 4.5-5.0 in mesophilic temperature ranges. The rise of antibiotic-resistant pathogenic strains dictates an increasing need for the survey of unexplored and underexplored niche habitats for novel antibiotic-producing actinomycetes strains.

Peat swamp forest is a special type of the evergreen forests that differs from other forests because it occurs in fresh-water marshy land. The soil in this forest is formed by a thick layer of peat, 0.5 to 5 meters deep, and has an acidic condition in which the range of soil pH is from 4.0 to 6.0. It may serve as a promising source of unexplored bioactive metabolite-producing actinomycetes. In Thailand, Thawai, *et al.* [8-9] isolated actinomycetes from peat swamp forest soils in Trang and Phatthalung Province, these strains were identified as *Streptomyces*, *Actinomadura*, *Micromonospora*, *Nocardia*, and *Actinoplanes*. Some of these strains exhibited antibacterial activity against *B. subtilis*, *S. aureus*, *E. coli*, and *P. aeruginosa*. However, all these isolates were neutrophilic actinomycetes. Antimicrobial activity of acidophilic actinomycete isolated from peat swamp forest of Thailand has never been reported before. Hence, the present study has been taken up with an objective of finding out the diversity of actinomycetes in peat soil collected from the Phatthalung Botanical Garden, Phatthalung Province, Thailand, and to estimate their potential to provide novel antibiotics.

## 2. Materials and Methods

### 2.1 Acidophilic actinomycete isolation

Peat soil samples were collected randomly from the depth of 15 cm from each location of Phatthalung Botanical Garden, Phatthalung Province, Thailand. The 10 grams of soil were each suspended in 90 ml sterile distilled water and heated in a water bath at 60 ºC for 15 min and was then serial diluted. Aliquots of 100 µl were spread on to Starch Casein Agar (SCA), Soil Extract Agar (SEA) and Humic Vitamin Agar (HVA), pH 4.5, containing 50 µg/ml nystatin and 50 µg/ml nalidixic acid, and incubated at 30 ºC for 7-21 days. The isolated colonies were picked up and streaked for purification on International Streptomyces Project (ISP) 2 agar medium. The strains were maintained as suspensions of mycelial fragments and spores in glycerol (20 %, v/v) at-80 ºC.

### 2.2 Screening of antagonistic activity

Antagonistic activity of acidophilic actinomycete isolates were tested by agar plug method. Actinomycetes were cultivated on ISP2 agar plate, pH4.5, at 30 ºC for 14 days. Agar plug were removed with a 6 mm diameter core from 14 days old cultures of the actinomycete from ISP2 agar medium. The agar plugs were placed onto the Nutrient agar plate which had been previously swabbed with one of the tested bacteria (*S. aureus* ATCC 25923, *B. cereus* TISTR 687, *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922) or fungi (*Fusarium* sp., *Curvularia* sp. and *C. gloeosporioides*) provided by department of plant pathology, faculty of agriculture, Kasetsart University, Thailand. The plates containing bacterial strains were incubated at 37 ºC for 24 h and the plates containing fungal strains were incubated at room temperature for 5 days. Following incubation, antimicrobial activity was indicated by the formation of an inhibition zone which provided an indication of diffused antimicrobial metabolites produced by the growing actinomycete culture [10].

### 2.3 Solvent extraction of antimicrobial compounds

Actinomycetes were grown in 0.7 liter of Yeast Malt broth, pH 4.5, contained in 2-liter flask at 30 ºC for 14 days with shaking (150 rpm). After incubation, the fermentation medium and mycelium were collected and filtered through Whatman No.1 filter paper. The total culture filtrate (500 ml) and mycelium were used for the series of solvent extractions by using hexane, ethyl acetate and methanol. Two folds volume of the solvent was mixed thoroughly with the broth by shaking them

in separating funnel and allowed to stand for 1 h. One hundred milliliter of solvent was mixed thoroughly with the mycelium and allowed to stand for 1 day. The solvents were pooled and removed by using simple distillation and vacuum rotary evaporator at 45 ºC. The crude extracts were dissolved in 5 ml of 20% DMSO and stored at 4 ºC until further use.

### 2.4 Antibacterial activity of crude extracts and Minimum Inhibitory Concentration (MIC)

The antibacterial activity of the crude extracts was tested on the 4 bacteria (*S. aureus* ATCC 25923, *B. cereus* TISTR 687, *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922) by agar well diffusion test. Tested bacteria were suspended in saline solution to a cell suspension of 0.5 McFarland. Bacterial suspension was spread on Mueller-Hinton agar (MHA – BBL, USA) plates and allowed to dry for 15 min. Then the wells (6 mm) were made on the inoculated plates using cork borer and each well was loaded with 30 µl of crude extract solution. The plates were incubated at 37°C for 24 h. Diameters of zones of inhibition were measured and recorded in millimeters [10]. The determination of the MIC of crude extracts was performed as described by the CLSI [11] with modifications. In 96-well plates, 95 µl of crude extracts and 95 µl of sterile Mueller-Hinton broth (MHB) were mixed in the first well, and the serial dilutions were carried out in all subsequent wells. To prepare the inoculum, the turbidity of the actively growing MHB culture was adjusted with sterile saline to obtain turbidity optically comparable to that of the 0.5 McFarland standard. The suspension was diluted 10-fold in sterile saline, and 5 µl of diluent was inoculated into 95 µl of MHB containing the crude extracts. The plates were incubated for 24 h at 37°C. The MIC was defined as the lowest concentration of an antibacterial agent that prevented visible growth under the test conditions.

### 2.5 Antifungal activity of actinomycete crude extracts

Determination of antifungal activities of actinomycete crude extracts was performed by using agar dilution method. Potato dextrose agar (PDA) plates were prepared and mixed with actinomycetes culture crude extracts of different concentrations with the following 7.5, 15, and 30 mg/ml. The plates were then each inoculated with an agar disc fungus (6 mm in diameter) being tested in the center of the Petri dish and incubated at 30°C for 5 days. Fungal growth (colony diameter) was measured and percentage inhibition calculated according to the formula: Percentage inhibition = [(C-T) x 100]/C. Where, C = colony diameter (mm) of the control; T = colony diameter (mm) of the test plate.

## 3. Results and Discussion

Acidophilic actinomycetes were isolated from peat soil samples from Phatthalung Botanical Garden, Phatthalung Province, Thailand, on SCA, SEA and HVA, pH 4.5, containing 50 µg/ml nystatin and 50 µg/ml nalidixic acid for inhibition of fungal and bacterial growth, respectively. The agar medium had been adjusted to pH 4.5 using citric acid buffer. The pH of the soil samples ranged from 4.2-4.6. The total acidophilic actinomycete counts ranged from 3.9-8.2 x $10^3$ CFU/g of soil sample. A total of 32 actinomycete colonies were selected randomly and made into pure cultures. Acidophilic actinomycetes can be assigned to two groups: neutrotolerant acidophilic strains grow from pH 4.5 to 7.5, with an optimum between pH 5.0 and 5.5; strictly acidophilic strains grow from pH 3.5 to 6.5, with an optimum around 4.5 [12-13]. Members of the latter group have been assigned to the genus *Streptacidiphilus*, which currently contains four species [5, 14]. Neutrotolerant acidophilic actinomycetes form a taxonomically diverse group within the range of variation encompassed by the genus *Streptomyces* [12-13], but only one species has a validly published name, *Streptomyces yeochonensis* [15]. However, all acidophilic actinomycetes used in this study have not been classified.

**Table 1.** Antagonistic activity of acidophilic actinomycetes isolated from peat soil samples

| Isolates No. | Inhibition zone (mm.) | | | | | | |
|---|---|---|---|---|---|---|---|
| | *B. cereus* | *S. aureus* | *P. aeruginosa* | *E. coli* | *Fusarium* sp. | *Curvularia* sp. | *C. gloeosporioides* |
| **AA01** | 13.0 | 10.8 | 14.3 | 8.2 | - | - | 11.0 |
| **AA02** | - | - | - | - | 16.0 | 17.0 | 16.5 |
| AA03 | - | - | - | - | - | 13.0 | 12.6 |
| AA04 | - | - | - | - | 13.0 | 14.5 | 15.2 |
| AA05 | 10.8 | - | - | - | - | 13.2 | 16.0 |
| AA06 | 9.0 | 10.8 | 12.3 | 8.2 | 14.2 | 13.4 | 15.5 |
| AA07 | - | - | - | - | - | - | - |
| AA08 | - | - | - | - | - | - | 12.0 |
| AA09 | - | - | - | - | 15.0 | 16.2 | 16.0 |
| AA10 | - | - | 15.0 | - | - | - | 12.2 |
| AA11 | 12.0 | 10 | 13.3 | - | - | - | - |
| AA12 | - | - | - | - | 14.5 | 15.2 | 15.6 |
| AA13 | - | - | - | - | - | 12.6 | - |
| AA14 | 15.7 | 12.9 | - | - | 13.0 | 17.0 | 15.8 |
| AA15 | 6.4 | - | 11.4 | - | 13.2 | 16.4 | 14.0 |
| AA16 | 7.6 | 7.8 | - | - | - | 15.6 | 15.2 |
| AA17 | - | - | - | - | - | - | - |
| AA18 | 14.6 | 8.5 | 8.4 | 7.8 | 16.2 | - | - |
| AA19 | - | - | - | - | - | - | - |
| AA20 | - | - | - | - | - | 14.4 | 12.6 |
| AA21 | - | - | - | - | - | - | - |
| AA22 | 9.3 | 10.4 | - | - | - | 15.5 | 14.4 |
| AA23 | 6.6 | - | - | - | 12.6 | 17.0 | 17.0 |
| AA24 | - | - | - | - | - | - | - |
| AA25 | 6.8 | 6.4 | - | - | 13.0 | 15.6 | 16.2 |
| AA26 | - | - | - | - | - | - | - |
| AA27 | - | - | - | - | 13.6 | 15.5 | 16.0 |
| AA28 | 9.8 | - | - | - | - | 16.2 | 14.8 |
| AA29 | 13.0 | 10.8 | 14.3 | - | - | - | - |
| AA30 | - | - | - | - | - | - | - |
| AA31 | - | - | - | - | - | 14.5 | 15.8 |
| AA32 | 12.0 | 8.8 | 12.2 | - | - | - | - |

*-, no inhibition zone*

In the present study, 32 acidophilic actinomycetes were isolated from acidic soils. All isolates were screened for antagonistic activity against 4 bacteria (*B. cereus*, *S. aureus*, *P. aeruginosa* and *E. coli*) and 3 fungi (*Fusarium* sp., *Curvularia* sp. and *C. gloeosporioides*). Of this, 14 strains (43.75%) inhibit *B. cereus*, 10 strains (31.25%) inhibit *S. aureus*, 8 strains (25%) inhibit *P. aeruginosa*, 3 strains (9.38%) inhibit *E. coli*, 11 strains (34.38%) inhibit *Fusarium* sp., 18 strains (56.25%) inhibit *Curvularia* sp., and 20 strains (62.5%) inhibit *C. gloeosporioides* (Table 1). Interestingly, 3 (9.38%) acidophilic actinomycete strains (AA01, AA06 and AA18) showed antibacterial activity against all 4 bacteria. The results revealed that more of the active

isolates were active against gram positive bacteria (*B. cereus* and *S. aureus*) than gram negative bacteria (*P. aeruginosa* and *E. coli*). The reason for different sensitivity between gram positive and gram negative bacteria could be ascribed to the morphological differences between these microorganisms. In general, Gram-negative bacteria are more resistant to chemical agents than the Gram-positive bacteria due to their outer membrane structures that act as a permeability barrier [16-17]. In Thailand, Thawai *et al*. [8-9] isolated actinomycetes from peat swamp forest soils in Trang and Phatthalung Province, which were identified as *Streptomyces*, *Actinomadura*, *Micromonospora*, *Nocardia*, and *Actinoplanes*. Some of these strains can inhibit *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC27853 and *Candida albicans* ATCC 10231. However, all these isolates were neutrophilic actinomycetes. Antimicrobial activity of acidophilic actinomycete isolated from peat swamp forest of Thailand has never been reported before.

In case of antifungal activity, 10 (31.25%) acidophilic actinomycete strains (AA02, AA04, AA06, AA09, AA12, AA14, AA15, AA23, AA25 and AA27) showed antifungal activity against all 3 fungi. Our finding is consistent with the study of Zakulyakina and Zenova [18] who showed that soil acidophilic actinomycetes markedly differ from neutrophilic actinomycetes in antimicrobial activity: the former are more active against fungi and yeasts, whereas the latter effectively suppress Gram-positive bacteria. Acidophilic streptomycetes actively inhibit the growth of phytopathogenic fungi, especially on acidic media [18]. Moreover, results of earlier studies [12, 19-20] show that acidophilic actinomycetes are spatially confined to acid soils. In such an environment, they are probably involved in competition with fungi, and, therefore, it is logical to assume that acidophilic actinomycetes possess an antifungal activity. Actinomycetes effective against phytopathogenic fungi have been searched for among conventional neutrophilic forms. In our view, acidophilic actinomycetes may be a productive source of new effective agents for controlling fungal plant diseases.

The potent actinomycetes were selected based on the results in preliminary screening for antagonistic activity. Strain AA01 and AA02 which inhibit all 4 tested bacteria and all 3 tested fungi, respectively, were selected as potent strains and used for fermentation and metabolite extraction. Both AA01 and AA02 are neutrontolerant acidophilic actinomycetes. They grew in media of pH levels ranging from 4.0 to 7.0; they grew well at pH 4.0-5.0 but poorly at pH 7.0. The crude extracts of AA01 culture prepared by organic solvent extraction (hexane, ethyl acetate and methanol) were analyzed for their antibacterial activity by agar diffusion method. In this study, methanolic extract showed good activity against all tested bacteria (Table 2) whereas ethyl acetate extract inhibited only *B. cereus*. There was no antibacterial activity observed in any hexane extract. The MIC values of methanolic crude extract from AA01 against *B. cereus*, *P. aeruginosa*, *E. coli* and *S. aureus* were 5, 20, 40 and 5 mg/ml, respectively.

**Table 2.** Antibacterial activity of crude solvent extracts of acidophilic actinomycete strain AA01

| Crude solvent extract of AA01 | Inhibition zone (mm.) | | | |
|---|---|---|---|---|
| | *B. cereus* | *S. aureus* | *P. aeruginosa* | *E. coli* |
| Hexane | - | - | - | - |
| Ethyl acetate | 16.4 | - | - | - |
| Methanol | 21.4 | 18.6 | 22.9 | 14.6 |

-, *no inhibition zone*

The crude extracts of AA02 were analyzed for their antifungal activity by agar dilution method. Only methanolic extract showed good activity against all 3 tested fungi. The inhibition of the mycelial growth of *Fusarium* sp., *Curvularia* sp. and *C. gloeosporioides* were 50.00, 63.36 and 75.17 %, respectively (Table 3 and Figure 1). There was no antifungal activity observed in hexane and ethyl acetate extracts. In this study, the methanolic extracts showed good activity against all the tested microorganisms (Tables 2 and 3). Isolation of an antibiotic from culture filtrate is largely determined by its chemical nature. Solvent extraction is usually employed for the extraction of antibiotics from the culture filtrates. Organic solvents with different polarities have been used by many researchers for extracting antimicrobial compounds from actinomycetes. This result clearly indicated that the antimicrobial activity of potent strains (AA01 and AA02) was due to the production of extracellular bioactive compounds.

**Table 3.** Antifungal activity of crude solvent extracts of acidophilic actinomycete strain AA02

| Crude solvent extract of AA02 | (%) Growth inhibition | | |
|---|---|---|---|
| | *Curvularia* sp. | *Fusarium* sp. | *C. gloeosporioides* |
| Hexane | 0 | 0 | 0 |
| Ethyl acetate | 0 | 0 | 0 |
| Methanol | 63.36 | 50.0 | 75.17 |

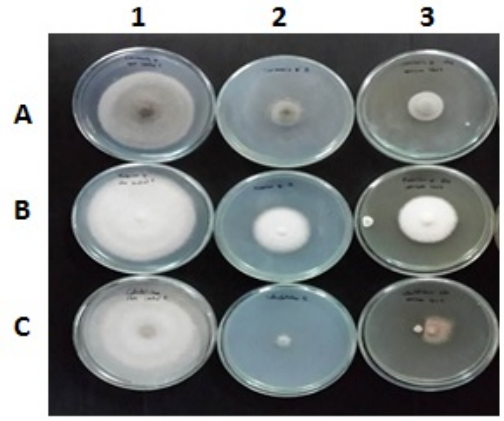


**Figure 1.** Antifungal activity of AA02 methanolic extract against Curvularia sp. (A), Fusarium sp. (B), and C. gloeosporioides (C). Row 1; PDA, row 2; PDA containing 50 µg/ml nystatin and row 3; PDA containing 30 mg/ml methanolic extract.

In conclusion, this finding is just a preliminary report. Antibiotics produced by these isolates should be further purified and analyzed and these acidophilic actinomycete isolates will be further systematically identified. The rise of antibiotic-resistant pathogenic strains dictates an increasing need for the survey of unexplored and underexplored habitats for novel antibiotic-producing strains. Acidic soil especially peat swamp forest soils in the Phatthalung Province, southern part of Thailand, may be an interesting source for screening of novel antibiotic-producing acidophilic actinobacteria.

## 4. Conclusions

Thirty-two acidophilic actinomycetes were isolated from peat soil samples collected from Phatthalung Botanical Garden and subjected to primarily screening for antimicrobial activity against tested bacteria and tested fungi. Three strains can inhibit all tested bacteria including gram positive and negative bacteria while 10 strains can inhibit all tested fungi. Strain AA01 and AA02 which inhibited all 4 tested bacteria and all 3 tested fungi, respectively, were selected as potent strains and used for fermentation and metabolite extraction. Ethyl acetate and methanolic extracts of AA01 strain showed promising antibacterial activity. Only methanolic extract of AA02 showed antifungal activity. Acidic soils especially peat swamp forest soils may serve as an interesting source for antibiotic-producing acidophilic actinobacteria.

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