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Actinomycetes from Rice Field Soil, Nakonsawan and Their Antimicrobial Activity

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ABSTRACT

A total of 100 actinomycetes isolates were isolated from rice field soil in Nong Bua, Nakonsawan, Thailand. All these strains were tested for preliminary and secondary screening for antimicrobial activity with test microorganisms including *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10231 and *Xanthomonas oryzae X3*. Partially purified extracellular and intracellular crude extracts were extracted using ethyl acetate and methanol as extraction solvents, respectively. Zones of inhibitions were obtained using disc diffusion method that contained 20 µl of partially purified extracellular and intracellular extraction samples (50 mg/ml and 1 mg/ml). The results showed that isolate NK13102 gave the highest inhibition zone in diameter against *B. subtilis* (26 mm), *S. aureus* (27 mm) *K. rhizophila* (29 mm) and *X. oryzae* (9 mm). Analysis of 16S rRNA gene sequence and blast analyses confirmed that the isolates belonging to the genus *Streptomyces* with similarity to *Streptomyces echinatus* (NK01105), *Streptomyces avellaneus* (NK13102), *Streptomyces bungoensis* (NK14101) and *Streptomyces laurentii* (NK17205).

Keywords: Actinomycetes, Rice field soil, 16s rRNA gene, Antimicrobial activity

INTRODUCTION

Potential use of microbes-based biocontrol-agents as supplement or replacements for agrochemicals has been reported in many recent reports (Shiva *et al.*, 2018) with the increased concern about conserving natural resources as soil, air and water, natural or biological control of plant diseases has received increased emphasis. Biological control of plant diseases is slow, gives few quick profits, but can be long lasting, inexpensive and harmless to life (Dhingra and Sinclair, 1995)

Rice paddy fields are unique ecosystems. Intensive rice cultivation was developed to increase yield and to meet the need of food security. This practice has many negative effects on the soil ecosystem, such as reduction of soil nutrients, soil and water pollution and increase in soil-borne plant pathogens and a possible reduction (Do, 2012)

Actinomycetes were accept as a wide attention by the reason of their potential as producers of many useful and several function bioactive secondary metabolites such as antibiotics and extracellular enzymes (Inbar *et al.*, 2005; Tarabily and Sivasithamparam, 2006). Actinomycetes have been associated as biocontrol agent in variously studies against plant pathogenic of fungi and bacteria (Baltz, 2008; Loqman *et al.*, 2009; Oskay, 2009). Filamentous bacteria belonging to the genus *Streptomyces* are well-known as the largest producer's antibiotic genus in the microbial world discovered so far (Taddei *et al*., 2006; Jayapal *et al*., 2007). approximately 80 % of the total antibiotics were produced by *Streptomyces* sp.

This study aims to isolate for new strains of actinomycetes that can produce antimicrobial activity and searching for potential to control *Xanthomonase oryzae* that causes bacterial blight (BB) in rice leaves. Identification of the active strains were done based on morphological properties, and physiological tests, in addition analysis of 16S rRNA gene sequencing were performed.

MATERIAL AND METHODS

Materials

Collection of soil sample

Soil samples were collected from rice field soil at Nong Bua, Nakonsawan, Thailand. Samples were randomised twenty-one point by collected the sediments 5-10 centimeters depth from the ground surface of

each location. These samples were kept in sterile polyethylene bags without sealed and transported immediately to the laboratory (Ruttanasutja and Pathomaree, 2015)

Isolation of Actinomycetes

These soil samples were pre-treated by air-dried samples for 3-5 days at room temperature. Ten grams of soil sample was accurately weighed and transferred to 90 mL of 0.1% Tween80 (10^{-1}), mixed well and then 1 mL of resultant solution was serially diluted with 0.1% Basic Lauryl Sulfate 9 mL up to 10^{-5}. 200 µl of each intermediate dilution was spread on Modified Zhang's Soil Starch Extract (ZSSE) agar medium that supplemented with nystatin (50 µg/ ml) . The plates were incubated for the growth of actinomycetes colonies at 30 °C and observed intermittently during incubation. After 7 days incubation, colony was picked by micro-needle then cross streak on International *Streptomyces* Project 2 (ISP2) medium. The pure colonies of actinomycetes isolates were selected, isolated and maintained on ISP2 medium at 30 °C for 7–14 days and preserved in 20% glycerol (w/v) stocks stored at -20 °C for long time preservation. (Waksman *et al*., 1961).

Morphological characterization of Actinomycetes isolates

All actinomycetes isolates were grown for 14 days at 30 °C on ISP2 agar medium and examined morphological by using light microscopy with long working distance lens (40X). Cultural and physiological characteristics were determined by using several standard methods; Cultural characteristics were performed by using 14 days cultures grown at 30 °C on ISP2 medium. The colour of aerial mycelium, substrate mycelium and soluble pigment were examined by using ISCC–NBS colour system (Kelly, 1964).

Antimicrobial activity

Preliminary screening for antimicrobial activity

The preliminary screening of the actinomycetes isolates against test microorganisms that including of *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10231 and *Xanthomonas oryzae X3* was performed by T' streak method. The T' streak method, ISP2 agar plates were used inoculated with the actinomycete isolate by single streak at the middle of petri dish plate and incubated at 30 °C for 7 days. The test microorganisms were

streaked perpendicularly to the actinomycetes and incubated (bacteria were incubated overnight at 30 °C and yeast at 37 °C for 48 h). The antagonism against each test microorganism was recorded in millimeter unit (Naif *et al.*, 2016).

Crude Extracts Preparation and Screening for Secondary metabolite

The actinomycetes strains which showed inhibitory activity form preliminary screening for antimicrobial activity were cultivated in ISP2 broth medium in a rotary shaker (150 rpm) at 30 °C for 14 days. To obtain the supernatant, the filtration of broth culture was done by using Whatman No. 1 filter paper. Using ethyl acetate as an organic solvent for extracting the bioactive compounds by adding an equal volume (v/v) of the solvent to supernatant filtrate in separation funnel for 3 times. The mixture of supernatant filtrate with ethyl acetate solvent was appearing in two layers, the organic solvent layer contained the secondary metabolites. The crude extract was obtained after evaporated solvent using rotary evaporator and stored in a desiccator. Harvested cells were fermented in equal volume of methanol for another 3 days and then also evaporated. The completely dried residues were dissolved in methanol to be used for further studies.

Agar disc diffusion

Antibacterial activity of partially purified extracellular (ethyl acetate crude extract) and intracellular (methanolic crude extract) crude extracts; were determined by agar disc diffusion method. Cell Concentration of all test microorganisms were adjusted at 0.5 McFarland turbidity standards and inoculated on Mueller Hinton Agar (MHA) for bacterial cells and Sabouraud Dextrose Agar (SDA) plates for yeast cell by using sterilized cotton swabs. Sterile disc containing 20 µl of each crude extract (concentration 1 and 50 mg/ml) were placed on the agar plates. Plates were incubated at 37°C for 24 h (Selvameenal *et al*., 2009).

Molecular identification

DNA amplification, sequencing and phylogenetic analysis of active isolates were identified by using 16S rRNA gene. The active isolates were grown on ISP2 agar medium for 4 days at 30 °C. The colony was picked up by a sterilized toothpick and resuspended in 40 µl of TE buffer pH 8.0 as DNA template. The 16S rRNA gene was amplified and sequenced by using primers 9F (5′ GAG TTTGATCITIGCTCAG3′) and 1541R (5′AAGGAGGTGA

TCCAGCC3′). The temperature for PCR amplification and sequencing reaction followed the method of Yukphan *et al*., (2005). Each PCR reaction of 50 µl in total included 25 µl AccuPower® *Taq* PCR Master Mix (Bioneer), 18 µl dH_2O, 2.5 µl the final concentration 10 pMol of each primer and 2 µl DNA template. The cycling conditions for the amplification of the 16s rRNA gene region were as follow: 3 mins at 94 °C, 25 cycles at 94 °C for 1 min, at 50 °C for 1 min and 2 min at 72 °C, then followed by a final extension step for 3 min at 72 °C. The sequences of 16s rRNA gene was aligned with the program BioEdit Sequence Alignment Editor (version 7.0.0. Distance matrices for the aligned sequences were calculated by using the two-parameter method of Kimura (1980). The neighbour-joining method Kumar *et al*., (2016) was used to construct a phylogenetic tree with the program MEGA7.

RESULTS

Isolation of Actinomycetes

A total of 100 actinomycetes isolates were isolated from rice field soil Nong Bua, Nakonsawan, Thailand. All these strains were collected by using Zhang's starch soil extract (ZSSE) agar medium supplemented with nystatin (50µg/ml). Cultural characteristics were performed using 14-day cultures grown at 30°C on ISP2 agar medium. The physiological and biochemical results are indicated of actinomycete strains, formed abundant, extensively branched substrate and aerial hyphae (Figure 1) were observed directly on the agar. The colour of aerial mycelium and substrate mycelium was examined using the ISCC–NBS Colour Charts standard sample (Kelly., 1964) into 13 groups; greyish-yellow, yellow, pale pink, pale purple, white, moderate olive brown, light green, strong yellowish brown, pale yellow green on ISP2 agar.

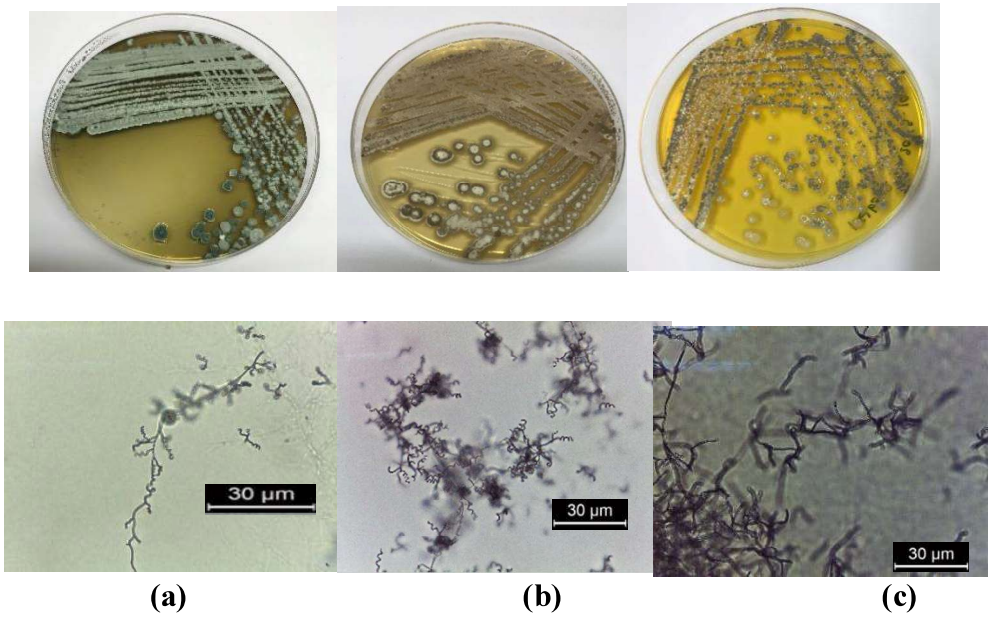

Figure 1. Morphological appearance colonies and spore arrangement of some actinomycetes isolates NK16103 (a), NK10101 (b) and NK16302 (c) on ISP2 agar medium .

Antimicrobial activity

Preliminary screening for antimicrobial activity of 100 isolates were tested against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC9341, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10231 and *Xanthomonas oryzae X3* using T'streak method. The isolates that gave positive result (Figure 2.) in preliminary screening for antimicrobial activity were used for secondary metabolite screen using agar disc diffusion method eventually.

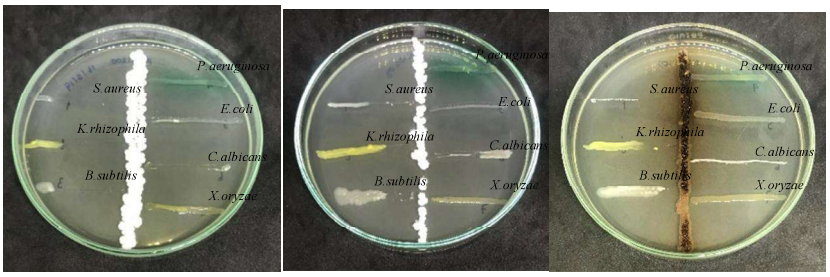

Figure 2. Some of actinomycetes showed antimicrobial activities against test microorganisms as evaluated by T' streak method. The central major streak corresponds to the actinomycetes isolates NK21200 (a), NK15203 (b) and NK01105 (c) and minor streaks with different microorganisms.

Crude Extracts Preparation and Screening for Secondary Metabolite Using Agar Disc Diffusion

The results of partially purified extracellular and intracellular crude extracts showed that 4 actinomycetes isolates were able to inhibit growth of some test microorganisms NK13102 gave the highest inhibition zone against *B. subtilis* (26 mm), *S. aureus* (27 mm), *K. rhizophila* (29 mm) and *X. oryzae* (9 mm) as shown in Table 1 and 2 (Figure 3), respectively. Almost all of the purified extracellular and intracellular crude extracts had ability to inhibit gram positive bacteria rather than gram negative ones.

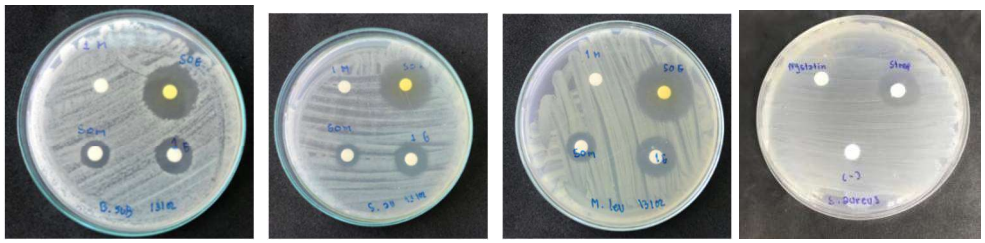

Figure 3. Antibacterial activitiy of the crude extract at 50 mg/ml and 1 mg/ml of isolate NK13102 against *B. subtilis* (a) *S. aureus* (b) *K.*

rhizophila (c), positive control 10µg/ml streptomycin and negative control as methanol (d) using disc diffusion method.

Table 1. Antibacterial activity of partially purified extracellular crude extracts produced by selected actinomycetes isolates.

Isolate No.	Disc Conc. (mg/ml)	Inhibition zone diameter (mm)						
		B.subtilis	*E.coli*	*K. rhizophila*	*P.aeru ginosa*	*S.aureus*	*C.albicans*	*X. oryzae*
NK01105	1	-	-	-	-	-	-	-
	50	15	-	13	-	11	-	-
NK13102	1	17	-	20	-	16	-	-
	50	26	-	29	-	27	-	9
NK14101	1	7	-	8	-	-		
	50	10	-	18	-	12		
NK17205	1	-	-	-	-	-	-	-
	50	-	-	-	-	-	-	-

Table 2. Antibacterial activity of partially purified intracellular crude extracts produced by selected actinomycetes isolates.

Isolate No.	Disc Conc. (mg/ml)	Inhibition zone diameter (mm)						
		B.subtilis	*E.coli*	*K. rhizophila*	*P.aerugin osa*	*S.aureus*	*C.albicans*	*X. oryzae*
NK01105	1	-	-	-	-	-	-	-
	50	10	-	10	-	8	-	-
NK13102	1	-	-	-	-	-	-	-
	50	13	-	14	-	10	-	-
NK14101	1	8	-	8	-	-	-	-
	50	10	-	17	-	12	-	-
NK17205	1	-	-	-	-	-	-	-
	50	9	-	12	-	10	-	7

Molecular identification

The 16S rRNA gene sequences and blast analyses confirmed that the active isolates belong to the genus *Streptomyces* with similarity of 99.37-99.86 %. These sequences were submitted to GenBank with accession number as showed in Table 3.

Table 3. Identification nucleotide sequences of actinomycetes using 16s \ rRNA gene

Isolate No.	Accession number	Identification	%similarity (Diff/Total nt)
NK01105	LC488880	*Streptomyces echinatus*	99.42 (8/1387)
NK13102	LC488884	*Streptomyces avellaneus*	99.51 (7/1436)
NK14101	LC488885	*Streptomyces bungoensis*	99.86 (2/1439)
NK17205	LC488888	*Streptomyces laurentii*	99.37 (9/1420)

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1362 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 that show in figure 4. (Kumar *et al*., 2016)

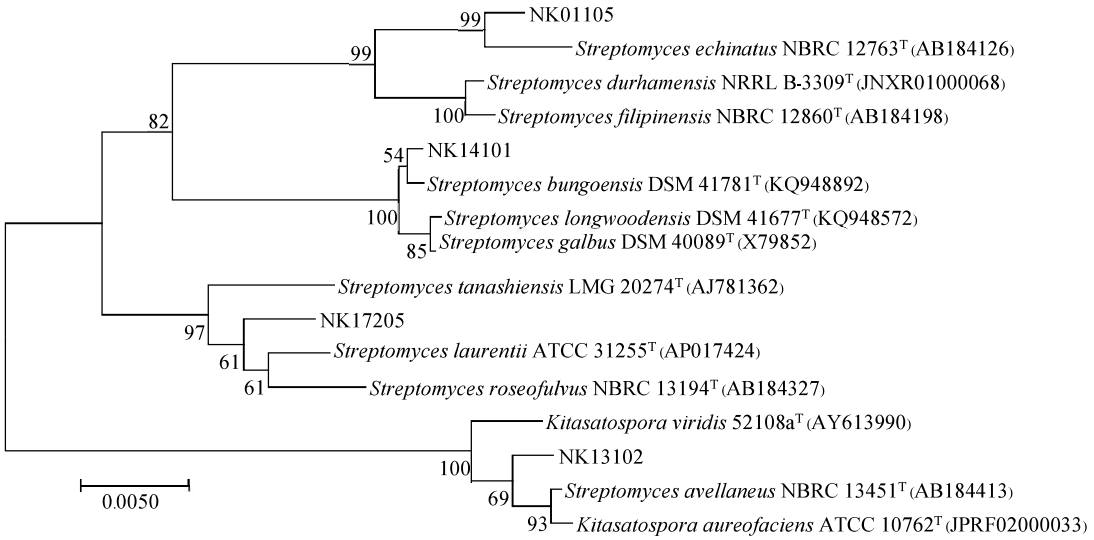

Figure 4. Phylogenetic tree based on 16S rRNA gene sequences using neighbour-joining method for 4 isolates of *Streptomyces* spp. and their closely related type strains.

DISCUSSION

Streptomyces is a soil- inhabiting, saprophytic genus well- known for production of antibiotics (Chater, 2006; Chater *et al.*, 2010). Most *Streptomyces* and other actinomycetes produce a diverse array of antibiotics including aminoglycosides, anthracyclins, glycol- peptides, β- lactams, macrolides, nucleosides, peptides polyenes, polyethers and tetra- cyclines. They produce about 75% of commercially and medically useful antibiotics (Mellouli *et al.*, 2003; Sahin and Ugur, 2003). The results in this study actinomycetes inhibit gram positive rather than gram negative as shown in previously literature by Ximin and Jun, (2013), production of beta-lactamases in gram negative bacteria these enzymes degrade beta- lactam antibiotics is the most widespread and threatening mechanism of antibiotic resistance. Identification of active isolates the result related to previously reports such as isolate NK01105 that tend to be *Streptomyces echinntus*. Gauvreau and Warin, (1984) reported that *Streptomyces echinntus* A8331 normally produces a single antibiotic, echinomycin (quinomycin A). Echinomycin is powerfully

active against experimental tumors and can be assayed by its activity against Gram- positive bacteria. Isolate NK13102 that tend to be *Streptomyces avellaneus*. Tsuyoshi *et al*., (1989) reported that *Streptomyces avellaneus* 02-3 produced novel chromomycin antibiotics, 02- 3G in addition several known related antibiotics. Isolate NK14101 that tend to be *Streptomyces bungoensis*. Jinhua *et al*., 2013 reported that *Streptomyces bungoensis* MJM 2077 Jinhua was selected for its strong anti- Methicillin- resistant *Staphylococcus aureu*/ Vancomycin- resistant *Enterococcus* activity and designated as *Streptomyces bungoensis*.

CONCLUSION

In this study, 100 actinomycetes were isolated from rice field soil Nong Bua, Nakonsawan, Thailand. Antimicrobial activity test of each crude extract was determined against test microorganisms. Isolate NK13102 gave the highest inhibition zone against *B. subtilis* (26 mm), *S. aureus* (27 mm) and *X. oryzae* (9 mm). Molecular identification of active isolates reveled their identities as the genus *Streptomyces* sp. with similarity to *Streptomyces echinatus* (NK01105), *Streptomyces niveiscabiei* (NK04101), *Streptomyces avellaneus* (NK13102), *Streptomyces bungoensis* (NK14101) and *Streptomyces laurentii* (NK17205).

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