Journal of Applied Pharmaceutical Science Vol. 3 (10), pp. 011-016, October, 2013
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2013.31003
ISSN 2231-3354

Characterization and antimicrobial activity of *Amycolatopsis* strains isolated from Thai soils

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ARTICLE INFO

Article history:
Received on: 07/09/2013
Revised on: 29/09/2013
Accepted on: 15/10/2013
Available online: 31/10/2013

Key words:
Actinomycetes, *Amycolatopsis*, antimicrobial activity, soil.

ABSTRACT

The isolation and screening of antimicrobial activity of 3 actinomycete strains isolated from soil samples collected in Chaiyaphum, Nan and Phatthalung provinces, Thailand were carried out. Strains S39-7, KC19-1 and K57-1 were belonged to the genus *Amycolatopsis* based on their phenotypic and chemotaxonomic characteristics. On the basis of 16S rRNA gene sequence analysis, strain S39-7 was closely related to *Amycolatopsis albidoflavus* KCTC 9471^T (99.2%). Strains KC19-1 and K57-1 were closely related to *A. kentuckyensis* NRRL B-24129^T with 99.3 and 99.2% similarity, respectively. All of them contained *meso*-diaminopimelic acid (DAP) in cell wall peptidoglycan and had MK-9 (H_4) as a major menaquinones. The DNA G+C contents of the strains ranged from 67.2 to 73.4 mol%. On secondary screening of antimicrobial activity, the ethyl acetate extract of the fermentation products of strain S39-7 was active against *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Kocuria rhizophila* ATCC 9341, and *Pseudomonas aeruginosa* ATCC 27853 while strain KC 19-1 was active against only *S. aureus* ATCC 6538. Strain K 57-1 was active against *E. coli* ATCC 25922 and *K. rhizophila* ATCC 9341. In addition strain S39-7 could inhibit against methicillin resistant (MRSA) *S. aureus* 266.

INTRODUCTION

The genus *Amycolatopsis* was established by Lechevalier *et al*., (1986) and was assigned to family *Pseudonocardiaceae* (Embley *et al*., 1988; Cross, 1994; Warwick *et al*., 1994). Recently, increasing interest has been shown in *Amycolatopsis* strains because they are a very important genus in the antibiotics industry. They produce some of the most widely used antibiotics such as rifamycin that produced from *A. mediterranei* (Meja *et al*., 1997) and vancomycin from *A. orientalis* (Pittenger and Brigham, 1956). In addition, vancoresmycin was produced from *A. vancoremycina* (Hopmann *et al*., 2002), balhimycin from *A. balhimycina*, tolypomycin from *A. tolypomycina* and nogabecin from *A. keratiniphila* (Wink *et al*., 2003), and decaplanin from *A. decaplanina* (Wink *et al*., 2004). Among them, rifamycim is one of the major drugs for clinical treatment of HIV-related tuberculosis, and vancomycin is currently considered as the last line of those defense against some microorganisms that are resistant to β- lactam antibiotics (Yao *et al*., 2002). In the course of our investigation of actinomycete isolates from soils in Thailand, the isolation and screening of antimicrobial activity and identification of strains were determined based on the phenotypic and chemotaxonomic characteristics including 16S rRNA gene sequence analysis.

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MATERIALS AND METHODS

Isolation and Characterization of the isolates

Three actinomycete strains were isolated from soil samples collected from Chaiyaphum, Nan and Phatthalung provinces, Thailand (Table 1) using starch-casein nitrate agar (Thawai *et al*., 2004). The phenotypic characteristics were determined by the methods described by Shirling and Gottlieb (1966) and Arai *et al*. (1976).

Scanning electron microscope was used for determining the morphology of strains grew on YMA (ISP medium no. 2, Yeast extract- Malt extract agar). Cell wall diaminopimelic acid (DAP) isomers were determined as described by Kutzner (1981). Menaquinone system was analysed as described by Komagata and Suzuki (1987).

16S rDNA sequence and phylogenetic analyses

DNA of the strains was isolated from cells grown in Yeast extract-Malt extract broth (YMB) with 0.2% of glycine reported by Yamada and Komagata (1970) and purified as described by Saito and Miura (1963). DNA base composition analysis was analysed by the method of Tamaoka and Komagata (1984). The complete 16S rRNA gene was amplified by PCR using primers, 8-27f and 1492r.

The amplified 16S rRNA gene was used as templates for sequencing with Big Dye Terminator sequencing Kit (Perkin Elmer) and analyzed by AB1377 automated DNA sequencer (Perkin Elmer). The sequencing reaction for each sample was performed in DNA Thermal Cycler (Gene Amp PCR System 2400; Perkin Elmer) by using primers, 8-27f (5'-AGAGTTTGATC (A/C)TGGCTCAG-3'), 530f (5'-GTGCCAGC(A/C)GCCGCGG-3') and 1114f (5'-GCAACGAGCGCAACCC-3'). Homology search was performed using the standard BLAST sequence similarity searching program version 2.2.1 from the web server. http://www.ncbi.nlm.nih.gov/BLAST/ against previously reported sequence at the GenBank/EMBL/DDBJ database. The sequence was multiply aligned with selected sequences obtained from GenBank/EMBL/DDBJ by using the CLUSTAL_X (Thompson *et al*., 1997).

The alignment was manually verified and adjusted prior to the construction of phylogenetic tree. The phylogenetic tree was constructed by using neighbor-joining (Saitou and Nei, 1987) in the MEGA program version 2.1 (Kumar *et al*., 2001). The confidence values of branches of the phylogenetic tree were determined using the bootstrap analyses based on 1000 resamplings (Felsenstien, 1985). 16S rDNA sequence of *Micromonospora chalcea* JCM 3082^T was used as an out group. The values for sequences similarity among the closest strains were calculated manually after pairwise alignments obtained using the CLUSTAL_X program (Thompson *et al*., 1997). Gaps and ambiguous nucleotides were eliminated from calculations.

Antimicrobial activity of strains

Primary screening of antimicrobial activities was performed on YMA plates (Anansiriwattana *et al*. (2006) against *S. aureus* ATCC 6538, *B. subtilis* ATCC 6633, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *Kocuria rhizophila* ATCC 9341 and *C. albicans* ATCC 10231. All tested microorganisms were cultivated on Mueller-Hinton agar slants at 37°C for 24 h, except for the yeast strain that was cultivated on Sabouraud's dextrose agar slant at 30°C for 24 h. Secondary screening of the strains was examined by cultivating each strains into a 500-ml Erlenmeyer flask containing 250 ml of YM broth and incubated on a rotary shaker at 200 rpm, 30°C for 11 days. The culture broth was extracted with ethyl acetate (EtOAc) and concentrated under reduced pressure to yield the crude extract. The ethyl acetate extracts were tested by agar disc diffusion method (Lorian, 1991).

RESULTS AND DISCUSSION

Isolation and characterization of isolates

Three soil samples were collected from Chaiyaphum, Nan and Phatthalung provinces, Thailand. Actinomycetes were isolated and cultivated on YMA and kept in cold room at 4 °C. Sources of samples, pH and strain number were shown in Table 1.

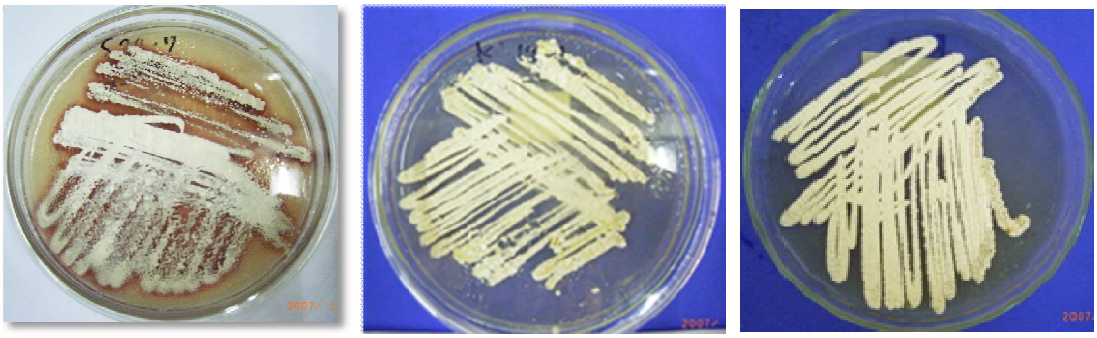
The strains S39-7, KC19-1 and K57-1 produced branched, fragmenting aerial and substrate mycelium with pink white, brownish white and yellowish white colonial color on YMA plates, respectively. They produced hyphae, spores borne in chains that are resemble to *Streptomyces* (Goodfellow *et al.*, 1988; Cross, 1994) (Figure 1). The cultural characteristics of strains on YMA, tyrosine agar, oatmeal agar, glycerol-asparagine agar and inorganic salt-starch agar are shown in Table 2.

Strains S39-7, KC19-1 and K57-1 grew on YMA with 2% and 4% NaCl, at pH 7.0, 9.0 and 10 and at 28°C, whereas only strain S39-7 could grow on 6% NaCl. They grew at pH and temperature within the range as reported previously (Cross, 1994). All strains could not form melanin. The physiological and biochemical characteristics of strains S39-7, KC19-1 and K57-1 were shown in Table 3.

All strains hydrolysed esculin, produced acid from adonitol, cellobiose, dextrin, *meso*-erythriol, fructose, glucose, D-galactose, *meso*-inositol, lactose, maltose, D-mannitol, melezitose, melibiose, methyl D-glucoside, raffinose, sucrose, trehalose and xylose; and utilized fructose, glucose, glycerol, D-mannitol, raffinose, rhamnose and xylose. Variable characteristics of strains were found in gelatin and starch hydrolysis, growth at pH 5, growth on 6% NaCl, acid production from L- arabinose, rhamnose, salicin and sorbitol; utilization of L- arabinose and melibiose (Table 3).

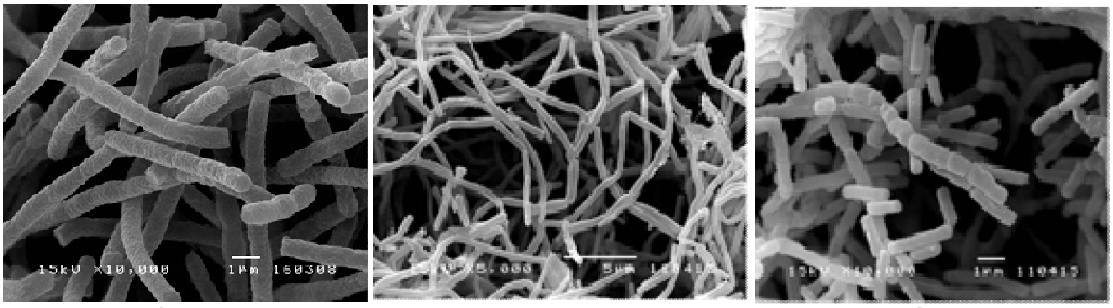
Strains KC19-1 and K57-1 grew on YMA containing 50 µg/ml and 100 µg/ml of novobiocin whereas strain S39-7 did not grow on YMA containing 50 µg/ml and 100 µg/ml of novobiocin comparison to *A. keratinophila* KCTC 19104^{T}and *A. albidoflavus* KCTC 9471^T that were sensitive to novobiocin 100 µg/ml (data not shown). However, other *Amycolatopsis* species such as *A. eurytherma* DSM 44348^T, *A. palatopharyngis* 1BDZ ^{T} and *A. rubida* JCM 10871 ^{T} were resistant to only on novobiocin 5 µg/ml (Huang *et al*., 2001; 2004; Kim *et al*., 2002). Therefore, the use of novobiocin in the medium for the screening of *Amycolatopsis* strains should be considered (Tajima *et al.*, 2001; Takahashi and Omura, 2003).

On the basis of cell wall peptidoglycan, the strains S39-7, KC19-1 and K57-1 contained *meso*-diaminopimelic acid which was the same pattern as the genus *Amycolatopsis*. The predominant menaquinone was MK-9 (H_4) and the small amounts of MK-9 (H_2), MK-9 (H_6), and MK-9 (H_8) were found. Their DNA G+C content ranged from 67.2-73.4 mol% as reported by Lechevalier *et al*. (1986) (Table 3).



A B C

Fig. 1: Colonial appearance of *Amycolatopsis* strains S39-7 (a), KC19-1 (b) and K57-1 (c).



A B C

Fig. 2: Scanning electron micrograph of *Amycolatopsis* strains S39-7 (a), KC19-1 (b) and K57-1 (c).

Table. 1: Location, pH of soil, strain number, 16S rRNA gene sequence similarity (%) and closest species

Location (Province)	pH	Strain no.	Similarity (%)	Closest species
Phatthalung	7.0	S39-7	99.2	*A. albidoflavus* KCTC 9471^T
Nan	6.8	KC19-1	99.3	*A. kentuckyensis* NRRL B-24129^T
Chaiyaphum	7.5	K57-1	99.2	*A. kentuckyensis* NRRL B-24129^T

Table. 2: Cultural characteristics of *Amycolatopsis* strains S39-7, KC19-1 and K57-1.

Strain no.	Medium	Growth	Spore color	Colony color	
				Upper colony	Lower colony
S 39-7	YM	+++	Pinkish white	Pinkish white Pinkish	Vivid purplish red
	Tyrosine	+++	Pinkish white	White	Dark violet
	Oatmeal	+++	Pinkish white	Pinkish white	Deep purplish red
	Asparagine	+++	Pinkish white	Pinkish white	Dark brown
	Inorg. salt	++	Pinkish white	Pinkish white	Vivid purplish red
KC 19-1	YM	+++	Brownish white	Brownish white	Pale beige
	Tyrosine	+++	Yellowish white	Yellowish white	Pale yellow
	Oatmeal	+++	Yellowish white	Yellowish white	Pale yellow
	Asparagine	+++	Brownish white	Brownish white	Pale beige
	Inorg. salt	+++	Brownish white	Brownish white	Pale beige
K 57-1	YM	+++	Yellowish white	Yellowish white	Pale beige
	Tyrosine	+++	Yellowish white	Yellowish white	Pale yellow
	Oatmeal	+++	Yellowish white	Yellowish white	Pale beige
	Asparagine	+++	Yellowish white	Yellowish white	Pale yellow
	Inorg. Salt	+++	Yellowish white	Yellowish white	Pale yellow

YMA, Yeast extract- Malt extract agar; Tyrosine, Tyrosine agar; Oatmeal, Oatmeal agar (Difco); Asparagine, Glycerol-Asparagine agar; Inorg. Salt, Inorganic salt-Starch agar. +++, good growth; ++, moderate growth.

16S rRNA gene sequence and phylogenetic analyses

Phylogenetic analysis of strains S39-7, KC19-1 and K57-1 revealed that they were belonged to the genus *Amycolatopsis* (Fig. 2). The percentage of 16S rRNA gene sequence similarity of *Amycolatopsis* strains to another strains were showed in Table 1. Strain S39-7 was closely related to *A. albidoflavus* KCTC 9471^T (Lee and Hah, 2001). The two organisms shared 16S rDNA similarity value of 99.2%. The strains KC19-1 and K57-1 were 99.3% related to each other and showed 99.3% and 99.2% similarility with *A. kentuckyensis* NRRL B-24129^T (Labeda *et al*., 2003), respectively. Strain S39-7 could produce dark red soluble pigment and produced acid from raffinose but no growth at 10 ° C. These characteristics could differentiate it from *A. albidoflavus* KCTC 9471^T (Lee and Hah, 2001). Strain KC19-1 could produce acid from raffinose but not decompose gelatin while K57-1 could produce acid from raffinose but not from L-arabinose and did not liquefy gelatin that differentiated them from *A. kentuchyensis* NRRL B-14129^T (Labeda *et al*., 2003). DNA-DNA hybridization experiment is acknowledged as the superior method for the elucidation of relationships between closely related taxa,

such as known strains and species, in which a DNA homology value of about >70% plays a dominant role (Wayne *et al*., 1987). For further study, *Amycolatopsis* strains S39-7, KC19-1 and K57-1 should be hybridized with closely related type strains for proposed that they are possible new species.

Antimicrobial activity of strains

Strain S39-7 exhibited antimicrobial activity against *S. aureus* ATCC 6538, *B. subtilis* ATCC 6633, *E. coli* ATCC 25922, *K. rhizophila* ATCC 9341 and *P. aeruginosa* ATCC 27853 while Strain KC 19-1 exhibited antimicrobial activity against only *S. aureus* ATCC 6538 and strain K 57-1 exhibited antimicrobial activity against *E. coli* ATCC 25922 and *K. rhizophila* ATCC 9341. All strains did not showed activitiy against *C. albicans* ATCC 10231 (Table 4).

In addition strain S39-7 could inhibit against methicillin resistant *S. aureus* (MRSA) 266. The study of antimicrobial substances from *Amycolatopsis* strains was interesting to further studies on the fermentation, extraction, purification, and structure elucidation.

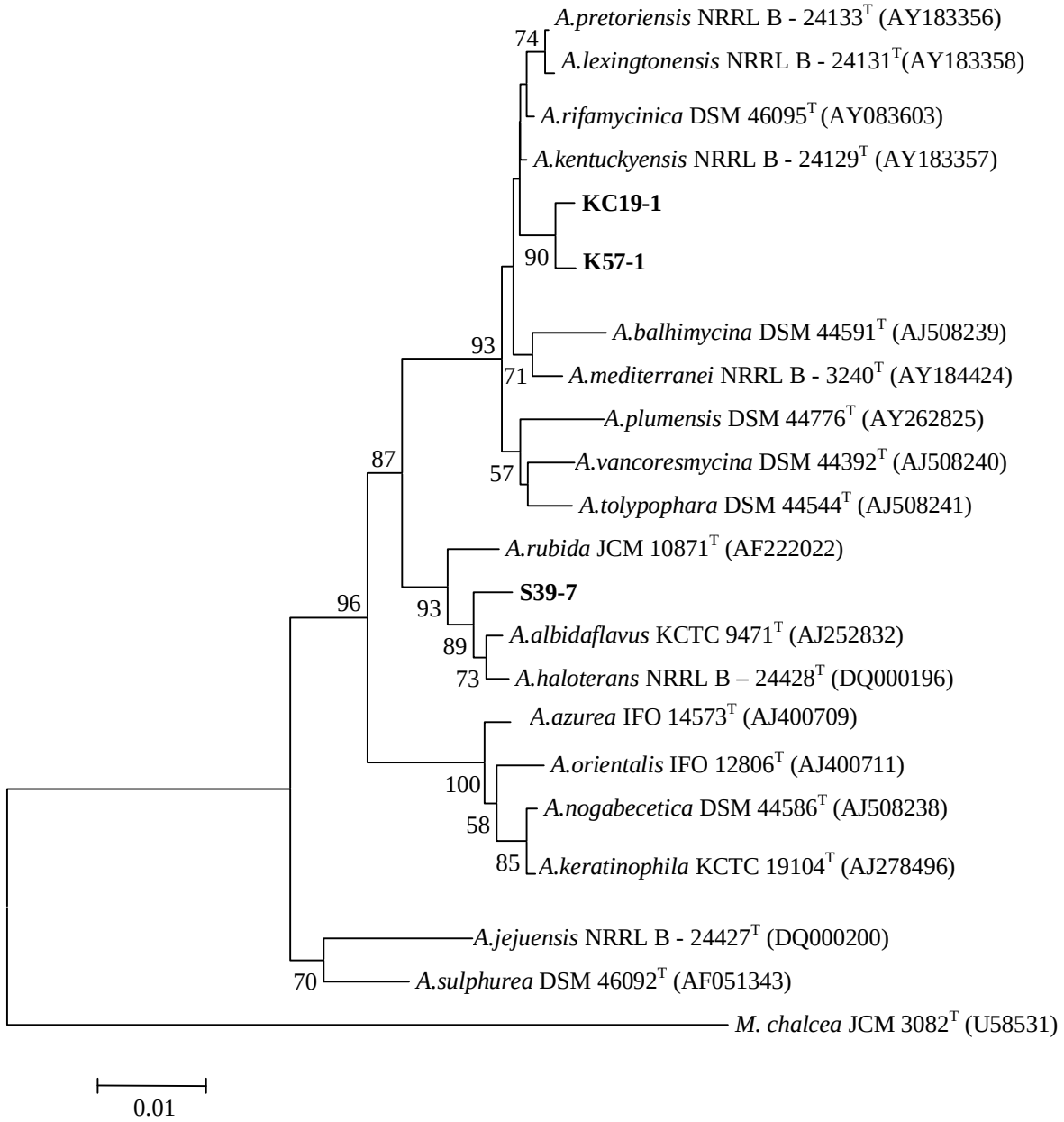


Fig. 3: Neighbor-joining tree showing the position of S39-7, KC19-1, K57-1 and the type strains of *Amycolatopsis* species based on 16S rRNA gene sequences.

Table. 3: Characteristics of *Amycolatopsis* strains S39-7, KC19-1 and K57-1.

Characteristics	**S39-7**	**KC19-1**	**K57-1**
Soluble pigment	Dark red	-	-
Gelatin liquefaction	+	-	-
Esculin hydrolysis	+	+	+
Starch hydrolysis	-	-	+
Growth at pH 5	+	-	+
Growth at pH 7-10	+	+	+
Growth on 4% NaCl	+	+	+
Growth on 6% NaCl	+	w	-
Growth on NV 50 µg/ml	-	+	+
Growth on NV100 µg/ml	-	+	+
Acid from			
Adonitol	+	+	+
L-Arabinose	+	+	-
Cellobiose	+	+	+
Dextrin	+	+	+
meso-Erythriol	+	+	+
D-Fructose	+	+	+
D-Galactose	+	+	+
Glucose	+	+	+
meso-Inositol	+	+	+
Lactose	+	+	+
Maltose	+	+	+
D-Mannitol	+	+	+
Melezitose	+	+	+
Melibiose	+	+	+
Methyl D-glucoside	+	+	+
Raffinose	+	+	+
Rhamnose	-	+	+
Salicin	-	+	+
Sorbitol	-	+	+
Sucrose	+	+	+
Trehalose	+	+	+
D-Xylose	+	+	+
Utilization			
L-Arabinose	+	+	-
D-Fructose	+	+	+
Glucose	+	+	+
Glycerol	+	+	+
D-Mannitol	+	+	+
Melibiose	-	+	+
Raffinose	+	+	+
Rhamnose	+	+	+
Sucrose	+	+	+
D-Xylose	+	+	+
Major menaquinone, MK-9 (H_4) (%)	94.5	91.2	88.5
DNA G +C (mol%)	67.2	73.4	73.1

+, positive; w, weak positive; -, negative**.** NV, novobiocin (µg/ml) in YMA.

Table 4: Antibacterial activity of *Amycolatopsis* strains S39-7, KC19-1 and K57-1

Strain no.	**Inhibition zone (mm)**					
	***S. aureus* ATCC 6538**	***S. aureus* MRSA 266**	***B. subtilis* ATCC 6633**	***E. coli* ATCC 25922**	***K. rhizophila* ATCC 9341**	***P. aeruginosa* ATCC 27853**
S 39-7	19	14	22	20	22	9
KC 19-1	15	-	-	-	-	-
K 57-1	-	-	-	12	14	-

CONCLUSION

The actinomycete strains, S39-7, KC19-1 and K57-1 isolated from soil collected in Phatthalung, Nan and Chaiyaphum respectively, were identified as *Amycolatopsis* based on their phenotypic and chemotaxonomic characteristics including 16S rRNA gene analyses. They could inhibit Gram-positive bacteria, *S. aureus* ATCC 6538, *B. subtilis* ATCC 6633 and *K. rhizophila* ATCC 9341 and Gram-negative bacteria, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 but showed no inhibitory activity against *C. albicans* ATCC 10231.

ACKNOWLEDGEMENT

The authors thank Graduate School, Chulalongkorn University (2006) for a partial support research grant to P.S.

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How to cite this article:

Piyapat Sripairoj, Khanit Suwanborirux and Somboon Tanasupawat. Characterization and antimicrobial activity of *Amycolatopsis* strains isolated from Thai soils. J App Pharm Sci, 2013; 3 (10): 011-016.