

Identification and antimicrobial activities of *Streptomyces*, *Micromonospora*, and *Kitasatospora* strains from rhizosphere soils

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ABSTRACT

Rhizosphere soils are the major habitat of various bacteria, especially the actinobacteria. In this study, 17 actinomycetes were isolated from rhizosphere soil samples collected from the plants, including *Barringtonia racemosa*, *Albizia odoratissima*, *Spondia spinnata*, and *Azadirachta indica*. On the basis of 16S rRNA gene analysis (99.0%–100% similarity), the isolates were belonged to genera *Streptomyces* (10 isolates), *Micromonospora* (5 isolates), and *Kitasatospora* (2 isolates). They were identified as *Streptomyces sioyaensis* (2 isolates), *Streptomyces vietnamensis* (2 isolates), *Streptomyces bungoensis* (2 isolates), each of *Streptomyces psammoticus*, *Streptomyces purpurascens*, *Streptomyces hydrogenans*, *Streptomyces lucensis*; *Micromonospora schwarzwaldensis*, *Micromonospora chersina*, *Micromonospora terminalae*, *Micromonospora chaiyaphumensis*, *Micromonospora rhizosphaerae* and two isolates as *Kitasatospora putterlickiae*. On the results of antimicrobial activities, three isolates presented the good activities against *Candida albicans* ATCC 10231, and *Escherichia coli* ATCC 25922, while 10 isolates exhibited the good activities against *Staphylococcus aureus* ATCC 25923 and 11 isolates exhibited activities against *Bacillus subtilis* ATCC 6633. Among them, JA03 showed 98.95% similarity of 16S rRNA gene sequence to *S. psammoticus* NBRC 13971^T, this isolate might be the novel species of actinomycetes.

INTRODUCTION

Actinobacteria are Gram-positive filamentous microorganisms that occur in diverse habitats both in terrestrial and aquatic environments. They are widely distributed in soils and some are strict saprophytes, but some are parasitic or live in mutualistic associations with plants and animals. These organisms have received more attention in recent years as producers of antibiotics, enzymes, and other proteins (Berdy, 2005; Goodfellow and Williams, 1983; Goodfellow *et al.*, 1988; Inahashi *et al.*, 2011). Recently, actinomycetes were found in other habitats, such as plant tissues (Taechowisan and Lumyong, 2003), root

nodules (Trujillo *et al.*, 2006), and the plant roots (Kuncharoen *et al.*, 2019a; 2019b). The present study dealt with the isolation, identification, and antimicrobial activities of actinobacteria from plant rhizosphere soil of plants, including *Barringtonia racemosa*, *Albizia odoratissima*, *Spondia spinnata*, and *Azadirachta indica*. at Kokkhumpoon Forest in the Sam Phrao campus, Udonthani Rajabhat University.

MATERIALS AND METHODS

Samples collection and isolation methods

Actinomycete strains were isolated from plant rhizosphere soils of four plants, including *B. racemosa*, *A. odoratissima*, *S. spinnata*, and *A. indica*. at Kokkhumpoon Forest in the Sam Phrao campus, Udonthani Rajabhat University, Udonthani province, Thailand. The pretreatment of soil samples was done using an air dry at room temperature (37°C ± 2) for 2 days. The 10-fold dilution series to 10⁻³ were prepared using 1 g

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of soil sample suspended in 9 ml of sterile water. The suspension (0.1 ml) was spread on Humic acid-Vitamin (HV) agar plate (Hayakawa and Nonomura, 1978) and Arginine-Vitamin (AV) agar plate (Nonomura and Ohara, 1969) and then incubated at 30°C for 14 days. Nalidixic acid (25 mg/l) and cycloheximide (50 mg/l) were added into the medium to inhibit other bacteria and fungi, respectively. The colonies of actinomycete isolates were observed using a light microscope and they were purified and cultivated on ISP2 medium. The selected isolates were preserved by freezing at -80°C in 15% (v/v) glycerol solution and by lyophilization.

Identification methods

Phenotypic and genotypic characteristics

The isolates were identified based on morphological, cultural, physiological, and biochemical characteristics, including 16S rRNA gene sequence analyses. Morphological and cultural characteristics of the isolates were observed on the culture grown on ISP2 medium at 30°C for 14 days (Shirling and Gottlieb, 1966). The colour of upper colony, reverse colony, and soluble pigment were observed using the National Bureau of Standards/ the Inter-Society-Color-Council (NBS/ISCC) colour chart (Mundie, 1995). In addition, the spore morphology and aerial or substrate mycelium were observed using a light microscope and scanning electron microscope (JSM-5410LV, Japan).

The DNA was extracted from the cells as described by Tamaoka (1994). The 16S rRNA gene amplification was carried out using two primers 20F and 1500R as reported by Suriyachadkun *et al.* (2009). The polymerase chain reaction (PCR) product was purified and the nucleotides were sequenced using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), 518F (5'-CCAGCAGCCGCGGTAATACG-3'), 800R (5'-TACCAGG GTATCTAATCC-3'), 1492R (5'-TACGGYTACCTTGTTACGA CTT-3') (Lane, 1991) (Macrogen; Seoul, Korea). The results of The Basic Local Alignment Search Tool (BLAST) analysis were assessed using the EzBioCloud server (Yoon *et al.*, 2017).

Antimicrobial activities

The four different production media including 301 medium (2.4 g starch, 0.1 g glucose, 0.3 g peptone, 0.3 g meat extract, 0.5 g yeast extract, 0.4 g CaCO₃, 100 ml distilled water, pH 7.0); 54 medium (2 g soluble starch, 0.5 g glycerol, 1 g defatted wheat germ, 0.3 g meat extract, 0.3 g yeast extract, 0.3 g CaCO₃, 100 ml distilled water, pH 7.0-7.2); 51 medium (0.5 g glucose, 0.5 g corn steep powder, 1.0 g oatmeal, 1.0 g pharmedia, 0.5 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 1 ml/l trace metal solution; 100 ml tap water), and Yeast extract-Dextrose (YD) broth (1.0 g yeast extract and 1.0 g dextrose, 100 ml distilled water) (Sripreechusak *et al.*, 2013; 2014) were used. Each isolate was cultivated in YD broth as a seed culture for 4-7 days. The inoculum of seed (0.1 ml) was transferred to 10 ml of each the production medium incubated in a shaker (180 rpm) at 30°C for 7-14 days. The extract solution was collected by centrifugation (3,400 rpm) for 15 minutes after 10 ml of 95% ethanol was added into the culture broth and shaken (180 rpm) for 2 hours.

The antimicrobial activities were performed using agar-disc diffusion method (Qin *et al.*, 2009). The paper disc (8 mm) was soaked into the extract solution of each strain and air-dried. The discs were put onto the surface of the agar plates containing

the indicator strains. The antimicrobial inhibition zones (diameter, mm) were determined after incubation for 24 hours. The production medium without the culture was used as the negative control. Four bacterial strains used as the indicator strains are *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aerogenosa* ATCC 27853 and one yeast strain, *Candida albicans* ATCC 10231. The bacterial strains were cultivated and incubated at 37°C for 24 hours, while the yeast strain was incubated at 30°C for 48 hours.

RESULTS AND DISCUSSION

Isolation and identification of isolates

The isolates JA03, JP09, and JP15 from *B. racemosa* and KA03, KA26 and KP38 from *A. odoratissima* while strains MKA22, MKA49, MKA54, MKA56, MKP02, MKP14, MKP30, MKP31, and MKP33 from *S. spinnata* and SDP02 and SDA19 from *A. indica* collected in Udonthani province, Thailand were obtained as shown in Table 1. Seventeen isolates were belonged to

Table 1. Isolate number, isolation source and cultural characteristics of isolates.

| Isolate no. | Rhizosphere soil of plant | Colony colour | | Soluble pigment |
|-------------|---------------------------|------------------------------|--------------------------|-------------------------------|
| | | Upper colony | Reverse colony | |
| Group 1 | | | | |
| JA03 | <i>B. racemosa</i> | Yellowish white | Pale Yellow | - |
| JP09 | <i>B. racemosa</i> | Greenish Gray to White | Strong Greenish Yellow | - |
| KA03 | <i>A. odoratissima</i> | Very Greenish Blue to White | Strong Yellow | - |
| MKA22 | <i>S. spinnata</i> | Pale Green to White | Pale Yellowish Green | - |
| MKA54 | <i>S. spinnata</i> | Moderate Greenish Yellow | Pale Greenish Yellow | Light Yellow |
| MKA56 | <i>S. spinnata</i> | Pale Blue | Strong Yellow Brown | Light Yellow |
| MKP02 | <i>S. spinnata</i> | White | Dark Grayish Yellow | - |
| MKP14 | <i>S. spinnata</i> | Very Pale Green to White | Pale Yellowish Green | - |
| SDP02 | <i>A. indica</i> | Very Pale Green | Moderate Yellowish Green | Light Yellow Green |
| SDA19 | <i>A. indica</i> | Greenish White | Reddish Gray | Strong Orange |
| Group 2 | | | | |
| JP15 | <i>B. racemosa</i> | Dark Grayish Yellowish Brown | Deep Yellowish Brown | - |
| KP38 | <i>A. odoratissima</i> | Blackish Green | Light Grayish Brown | - |
| MKP30 | <i>S. spinnata</i> | Greenish Black | Very Dark Green | Light Yellow |
| MKP31 | <i>S. spinnata</i> | Greenish Black | Very Dark Green | - |
| MKP33 | <i>S. spinnata</i> | Blackish Green | Very Pale Green | - |
| Group 3 | | | | |
| KA26 | <i>A. odoratissima</i> | Pale Green | Very Dark Bluish Green | Light Grayish Yellowish Brown |
| MKA49 | <i>S. spinnata</i> | Greenish Gray | Deep Bluish Green | - |

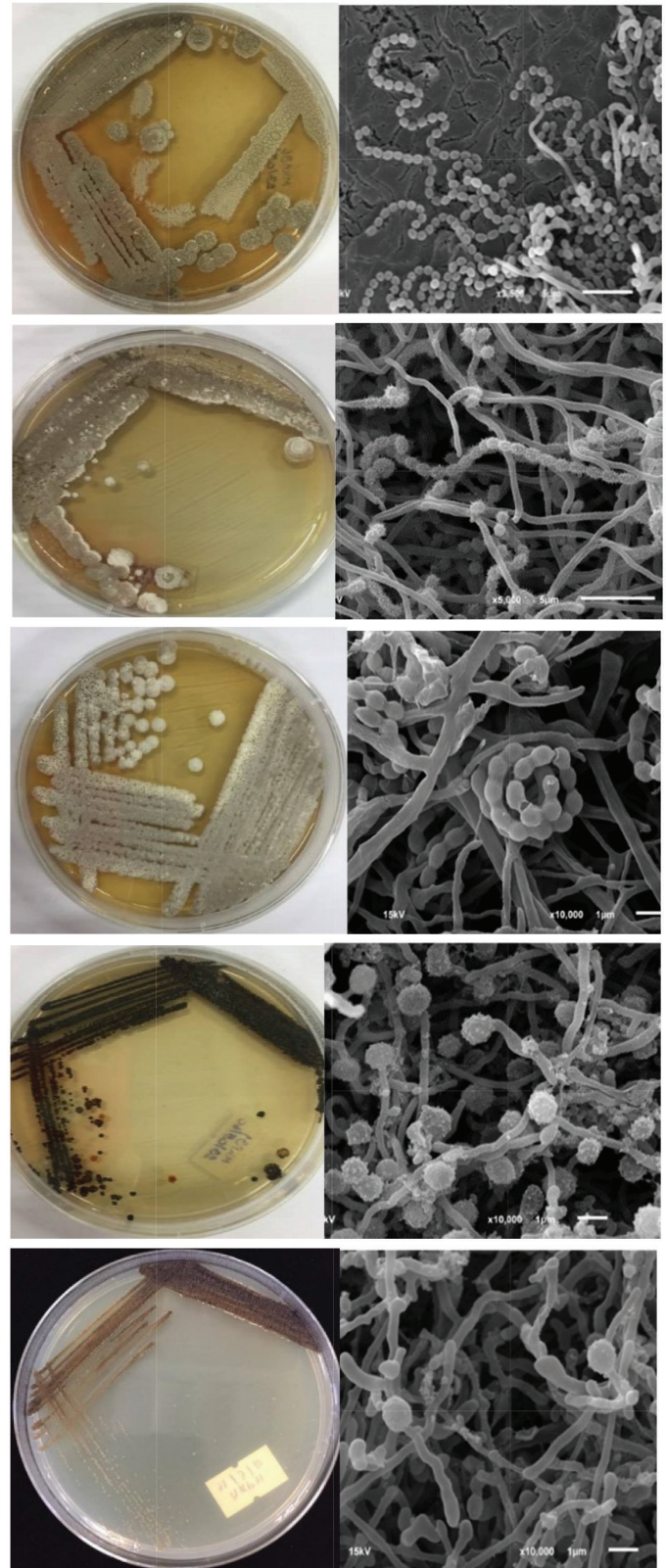
-, no activity

Table 2. Isolate number and nearest relatives of isolates based on 16S rRNA gene similarity (%).

| Isolate no. | Nearest relatives (% Similarity) |
|-------------|---|
| Group 1 | |
| JA03 | <i>S. psammoticus</i> NBRC 13971 ^T (98.95) |
| JP09 | <i>S. siyoaensis</i> NBRC 12820 ^T (100) |
| KA03 | <i>S. vietnamensis</i> CCTCCM 205143 ^T (99.98) |
| MKA22 | <i>S. vietnamensis</i> CCTCCM 205143 ^T (99.8) |
| MKA54 | <i>S. bungoensis</i> NBRC 15711 ^T (99.0) |
| MKA56 | <i>S. bungoensis</i> NBRC 15711 ^T (99.62) |
| MKP02 | <i>S. siyoaensis</i> NBRC 12820 ^T (99.8) |
| MKP14 | <i>S. purpurascens</i> NBRC 3389 ^T (99.77) |
| SDP02 | <i>S. hydrogenans</i> DSM 40586 ^T (99.62) |
| SDA19 | <i>S. lucensis</i> DSM 40317 ^T (99.23) |
| Group 2 | |
| JP15 | <i>M. schwarzwaldensis</i> DSM 45708 ^T (99.55) |
| KP38 | <i>M. chersina</i> DSM 44151 ^T (99.70) |
| MKP30 | <i>M. terminaliae</i> DSM 101760 ^T (99.70) |
| MKP31 | <i>M. chaiyaphumensis</i> TISTR 1564 ^T (99.26) |
| MKP33 | <i>M. rhizosphaerae</i> DSM 45431 ^T (99.05) |
| Group 3 | |
| KA26 | <i>K. putterlickiae</i> DSM 44665 ^T (99.26) |
| MKA49 | <i>K. putterlickiae</i> DSM 44665 ^T (99.85) |

the genus *Streptomyces* (10 isolates, Group 1), *Micromonospora* (5 isolates, Group 2), and 2 isolates were *Kitazatospora* (Group 3) based on their phenotypic characteristics and 16S rRNA gene sequence analysis as described below (Table 2).

Group 1 contained 10 isolates, JA03, JP09, KA03, MKA22, MKA54, MKA56, MKP02, MKP14, SDP02, and SDA19. They produced mature spore chains on ISP2 medium agar. Colonial appearance and scanning electron micrograph of isolates MKA56, MKA54, and SDP02 are shown in Figure 1. Variable characteristics of them were found in growth at pH 4–9 and on 0%–9% (w/v) NaCl, gelatin and starch hydrolysis, milk peptonization and coagulation, and nitrate reduction (Table 3). The isolates JA03, JP09, KA03, MKA22, MKA54, MKA56, MKP02, MKP14, SDA02, and SDA19 were belonged to the genus *Streptomyces* (Table 2) based on the phylogenetic analysis. Isolate JA03 was closely related to *Streptomyces psammoticus* NBRC 13971^T with 98.95% similarity that might be the novel species. Isolates JP09 and MKP02 were closely related to each other and showed 100% and 99.8% similarity to *Streptomyces siyoaensis* NBRC 12820^T. Isolates KA033 and MKA22 were closely related to each other and showed 99.98% and 99.8% similarity to *Streptomyces vietnamensis* CCTCCM 205143^T. Isolates MKA54 and MKA56 were closely related to each other and showed 99% and 99.62% similarity to *Streptomyces bungoensis* NBRC 15711^T. Isolate MKP14 were closely related to *Streptomyces purpurascens* NBRC 3389^T (99.77%). Isolate SAP02 was closely related to *Streptomyces hydrogenans* DSM 40586^T (99.62%), while isolate SDA19 was closely related to *Streptomyces lucensis* DSM 40317^T (99.23%). They were identified as *S. siyoaensis*, *S. vietnamensis*, *S. bungoensis*, *S. purpurascens*, *S. hydrogenans*, and *S. lucensis*, respectively (Zhu *et al.*, 2007).

**Figure 1.** Colonial appearance and scanning electron micrograph of isolates MKA56, MKA54, SDP02, MKP30, and MKP31.

Group 2 contained five isolates, JP15, KP38, MKP30, MKP31, and MKP33 (Table 1). They produced single spore on substrate mycelium on ISP2 medium agar at 30°C for 14 days

(Fig. 1). Their colonies color on ISP2 medium were varied from dark grayish yellowish brown to blackish green as described in Table 1. Variable characteristics of them were growth at pH 5–9, on 0%–5% (w/v) NaCl, gelatin and starch hydrolysis, nitrate reduction, milk peptonization, and coagulation (Table 3).

The isolates JP15, KP38, MKP30, MKP31, and MKP33 revealed that they were belonged to the genus *Micromonospora* based on the phylogenetic analysis (Kawamoto, 1989) (Table 2). Isolate JP15 was closely related to *Micromonospora schwarzwaldensis* (99.55%), while isolate KP38 was closely related to *Micromonospora chersina* (99.70%). Isolate MKP30 was closely related to *Micromonospora terminaliae* (99.70%). Isolate MKP31 was closely related to *Micromonospora chaiyaphumensis* (99.26%), while isolate MKP33 was closely related to *Micromonospora rhizosphaerae* (99.05%). They were identified as *M. schwarzwaldensis*, *M. chersina*, *M. terminaliae*, *M. chaiyaphumensis*, and *M. rhizosphaerae*, respectively (Gurovic *et al.*, 2013; Kaewkla *et al.*, 2017; Wang *et al.*, 2011).

Group 3 contained two isolates, KA26 and MKA49 (Table 1). They produced mature spore chains on ISP2 medium agar at 30°C for 14 days. Their colonies color on ISP2 medium were pale green and very dark bluish green, respectively (Table 1). The isolates grew at pH 4–9, on 3% (w/v) NaCl, and showed positive reaction for starch hydrolysis, peptonization, nitrate reduction, and milk coagulation

(Table 3). Isolates KA26 and MKA49 were closely related to *Kitasatospora putterlickiae* with 99.26% and 99.85% similarity based on the phylogenetic analysis, respectively. Therefore, they were identified as *K. putterlickiae* (Groth *et al.*, 2003).

Antimicrobial activities

Streptomyces isolates JA03 and MKA56 exhibited strong antimicrobial activity against *C. albicans* ATCC 10231, while isolates JP09, MKA54, MKP02, and MKP14 showed strong antimicrobial activity against *S. aureus* ATCC 25923 and *B. subtilis* ATCC 6633. *Micromonospora* sp. MKP30 exhibited strong antimicrobial activity against *S. aureus* ATCC 25923 and *B. subtilis* ATCC 6633 while *Kitasatospora* isolates KA26 and MKA49 exhibited against *S. aureus* ATCC 25923 and *B. subtilis* ATCC 663. Only isolates MKA22, MKA56, and KA26 exhibited strong antimicrobial activity against *E. coli* ATCC 25922. Fourteen strains did not showed antibacterial activity to *P. aeruginosa* ATCC 27853. The detailed antimicrobial activities are presented in Table 4.

In Thailand, a large number of *Streptomyces*, *Micromonospora*, and *Kitasatospora* strains were isolated from soils (Anansiriwattana *et al.*, 2006; Sripairoj *et al.*, 2008; Sriprechasak *et al.*, 2013; 2014). In addition, the strains of *Streptomyces* and *Micromonospora*, including the novel

Table 3. Phenotypic characteristics of isolates.

| Characteristics | Isolate no. | | | | | | | | | | | | | | | | |
|------------------------|-------------|------|------|-------|-------|-------|-------|-------|---------|-------|------|------|-------|---------|-------|------|-------|
| | Group 1 | | | | | | | | Group 2 | | | | | Group 3 | | | |
| | JA03 | JP09 | KA03 | MKA22 | MKA54 | MKA56 | MKP02 | MKP14 | SDP02 | SDA19 | JP15 | KP38 | MKP30 | MKP31 | MKP33 | KA26 | MKA49 |
| Max. NaCl (%w/v) | 4 | 8 | 9 | 4 | 8 | 5 | 8 | 6 | 9 | 4 | 5 | 4 | 4 | 4 | 3 | 4 | 3 |
| pH range for growth | 4–9 | 4–9 | 5–9 | 5–9 | 5–9 | 5–9 | 5–9 | 4–9 | 5–9 | 4–9 | 5–9 | 5–9 | 5–9 | 5–9 | 5–9 | 4–9 | 5–9 |
| Gelatin liquefaction | + | + | + | – | + | + | + | – | + | + | + | + | + | + | – | + | + |
| Nitrate reduction | + | + | – | – | + | – | – | – | – | + | – | – | – | – | + | + | – |
| Milk peptonization | – | – | – | + | + | – | – | – | + | + | – | – | – | + | – | + | + |
| Milk coagulation | – | + | + | + | + | + | + | + | + | + | + | – | + | + | + | + | + |
| Starch hydrolysis | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Utilization of: | | | | | | | | | | | | | | | | | |
| Arabinose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Cellobiose | + | + | + | + | + | + | + | – | + | + | + | + | + | + | + | + | + |
| Cellulose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Fructose | + | + | + | + | + | + | + | + | + | + | + | + | + | – | + | + | + |
| Galactose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Glucose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Glycerol | + | + | – | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Inositol | – | + | + | + | + | – | – | – | + | + | – | – | – | + | – | + | + |
| Mannitol | + | + | + | + | + | + | + | + | + | + | + | + | – | + | + | + | + |
| Mannose | – | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Melibiose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Raffinose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Rhamnose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Sucrose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Xylose | – | + | + | + | + | + | + | + | + | + | + | – | + | – | + | + | + |

+, positive reaction; –, negative reaction

Table 4. Antimicrobial activity of isolates.

| Isolate no. | Inhibitory against indicator strains (mm.) | | | | |
|-------------|--|----|---|------|----|
| | C | E | P | S | B |
| Group 1 | | | | | |
| JA03 | 15 | - | - | 12 | 13 |
| JP09 | 6 | 5 | - | 39 | 31 |
| KA03 | - | - | - | 4 | 32 |
| MKA22 | - | 20 | - | 15 | 10 |
| MKA54 | - | - | - | 45 | 40 |
| MKA56 | 26 | 18 | - | 0.5 | - |
| MKP02 | 9 | - | - | 40 | 53 |
| MKP14 | - | - | - | 43 | 50 |
| SDP02 | 10 | - | - | 10.5 | 10 |
| SDA19 | 4 | 4 | 5 | - | - |
| Group 2 | | | | | |
| JP15 | - | - | - | - | - |
| KP38 | - | - | - | - | - |
| MKP30 | - | - | 5 | 20 | 14 |
| MKP31 | 6 | - | - | - | 8 |
| MKP33 | - | - | - | - | - |
| Group 3 | | | | | |
| KA26 | 8 | 27 | - | 26 | 45 |
| MKA49 | - | - | - | 35 | 52 |

C, *C. albicans* ATCC 10231; E, *E. coli* ATCC 25922; P, *P. aeruginosa* ATCC 27853; S, *S. aureus* ATCC 25923; B, *B. subtilis* ATCC 6633; -, no activity.

species of *Micromonospora azadirachtae* and *Micromonospora globbae* strains from plant roots are reported (Kuncharoen et al., 2018; 2019a; 2019b). In this study, we found diverse species of *Streptomyces* including *S. psammoticus*, *S. siyoaensis*, *S. vietnamensis*, *S. bungoensis*, *S. purpurascens*, *S. hydrogenans*, *S. lucensis*, *Micromonospora*, *M. schwarzwaldensis*, *M. chersina*, *M. chersina*, *Micromonospora terminaliae*, *M. rhizosphaerae*, and *K. putterlickiae* from rhizosphere soils that showed antimicrobial activities and their secondary metabolites are interesting for further study.

CONCLUSION

Actinomycetes were isolated from rhizosphere soil samples collected from four plants including *B. racemosa*, *A. odoratissima*, *S. spinata*, and *A. indica*. They were identified as *S. psammoticus*, *S. siyoaensis*, *S. vietnamensis*, *S. bungoensis*, *S. purpurascens*, *S. lucensis*, *M. schwarzwaldensis*, *M. chersina*, *M. terminaliae*, *M. chaiyaphumensis*, *M. rhizosphaerae*, and *Kitasatospora putterlickiae* based on 16S rRNA gene sequence analysis. On the antimicrobial activities, the isolates exhibited the good activities against *C. albicans* ATCC 10231, *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *B. subtilis* ATCC 6633.

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CONFLICT OF INTERESTS

The authors declared that they have no conflicts of interest.

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