Screening and Identification of Rare Actinomycetes Isolated from Soil in Pho Hin Dad Waterfall (Namtok Sam Lan National Park, Saraburi Province) for Antimicrobial Activities

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Abstract

Actinomycetes are common soil microorganisms with antibiotic-producing potential. In this study, three actinomycete strains were isolated from the soil in Pho Hin Dad Waterfall, Namtok Sam Lan National Park, Saraburi province, Thailand. Based on phenotypic characteristics and 16S rRNA gene sequence analysis, these actinomycete strains (P9, P12 and P15) belonged to the genus *Dactylosporangium*, *Nonomuraea*, and *Actinomadura*, while most closely related to *Dactylosporangium sucinum* RY35-23^T (100%), *Nonomuraea jiangxiensis* CGMCC 4.6533^T (99.57%), and *Actinomadura barringtoniae* GKU 128^T (99.35%), respectively. Crude ethyl acetate extracts from strain P9, P12, and P15 showed antimicrobial activities against Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341, *Staphylococcus aureus* ATCC 25923, and Methicillin-resistant *Staphylococcus aureus* DMST 20654) and yeast (*Candida albicans* ATCC 10231), but not against Gram-negative bacteria *(Escherichia coli ATCC 25922* and *Pseudomonas aeruginosa* ATCC 27853). The result in this study demonstrated the antibiotic-producing potential of actinomycetes from the soil in Pho Hin Dad Waterfall.

Keywords**:** *Rare actinomycetes, Antimicrobial activity, Identification, 16S rRNA, Pho Hin Dad Waterfall*

1. Introduction

Actinomycetes are Gram-positive, filamentous bacteria with high G+C content in their DNA (>55%) (Stackebrandt et al., 1997). They are the biggest group of antibiotic producers (45%) among the microorganisms, followed by fungi (38%) and other bacteria (17%) (Berdy, 2005). Many of antibiotics from actinomycetes are important in medicine, such as actinomycin, chloramphenicol, erythromycin, kanamycin, neomycin, streptomycin and tetracycline (Goodfellow et al., 1988; Sharma et al., 2014). Among actinomycetes, *Streptomyces* is the largest antibiotic-producing genus that 70% of antibiotics are obtained, 30% of antibiotics remaining obtained from another genus which so-called rare actinomycetes (Berdy, 2005). Because of the serious problem of emerging antimicrobial resistance in recent years (Kmietowicz, 2017), the search for new antibiotics is importance. However, the discovery of novel compounds with antibacterial activities from *Streptomyces* was decreased due to the rediscovery of known antibiotics from them (Hug et al., 2018). Additionally, new antibiotics were more discovered from rare actinomycetes (Tiwari & Gupta, 2012). Therefore, more research interest was directed towards rare actinomycetes for the discovery of novel antibiotic compounds.

Actinomycetes are widely distributed in the environment, mainly in soil, of which one million actinomycete cells were found in one gram (Miyadoh, 1997). Despite the extensive study of soil actinomycetes, unexplored soil habitats remain potential sources for obtaining actinomycetes with antibiotic-producing potential.

Pho Hin Dad Waterfall is located in Namtok Sam Lan National Park in Saraburi province, Thailand. The surrounding areas are mixed deciduous and dry evergreen forests with a high abundance of various flora and fauna. The waterfall soil is highly fertile. Additionally, there were no reports of actinomycete isolation from the waterfall area. Therefore, the present study was done to isolate and identify actinomycete inhabiting the waterfall soil. Rare actinomycetes were obtained, and their antimicrobial activities were evaluated.

2. Objectives

To identify and evaluate antimicrobial activities of rare actinomycetes isolated from soil in Pho Hin Dad Waterfall, Saraburi province, Thailand.

3. Materials and Methods

3.1 Sample collection and Isolation of actinomycetes

The soil sample was collected from Pho Hin Dad Waterfall in Namtok Sam Lan National Park, Saraburi province, Thailand. The sample was dried at room temperature for 14 days. 1 g of dried sample was ground and suspended in 9 ml of sterile distilled water. The suspension was diluted to 10^{-5} by tenfold serial dilution. One-hundred µl of the solution (10^{-5}) was spread on humic acid-vitamin agar (Hayakawa & Nonomura, 1987) supplemented with 25 mg l^{-1} nalidixic acid, 50 mg l^{-1} nystatin and 1 mg l^{-1} terbinafine. After incubation at 30 °C for 30 days, the colonies of actinomycetes were cultured and purified on ISP2 agar (International *Streptomyces* project medium no.2) (Shirling & Gottlieb, 1966).

3.2 Identification of actinomycetes

Three actinomycete strains were identified, using phenotypic characteristics and 16S rRNA gene sequence analysis. Morphological characteristics were observed on modified soil extract agar (Suriyachadkun et al., 2009) at 30°C for 21 days by a light microscope with a 40X long working distance objective lens. Cultural characteristics were determined on ISP media (ISP2, ISP3, ISP4, ISP5, ISP6 and ISP7) (Shirling & Gottlieb, 1966), glucose-asparagine agar, Czapek's sucrose agar (Waksman, 1961) and nutrient agar (Microgen, India) at 30°C for 14 days. The colors of the colony and soluble pigment were determined using the ISCC-NBS Color Charts (Kelly, 1964). Gelatin liquefaction, peptonization of milk, nitrate reduction and starch hydrolysis were determined as described by Arai (Arai, 1975). Temperature, pH and NaCl tolerance were tested on ISP2 agar at 30°C for 14 days.

The actinomycete strains were cultured in ISP2 broth on a rotary shaker at 30 °C for 5 days. Cells were collected by centrifugation. Genomic DNA was extracted from cells by the method of Tamaoka (Tamaoka, 1994). The 16S rRNA gene was amplified using primers 9F (5'-GAGTTTGATCCTGGCTCAG -3') and 1541R (5'-AAGGAGGTGATCCAGCC-3'). The PCR reaction mixture (100 µl) contained 10 µl of 10X PCR reaction buffer, 8 µl of 25 mM $MgCl_2$, 8 µl of 2 mM dNTP mixture, 5 µl each of primer (10 µM), 0.5 µl of *Taq* DNA polymerase (Invitrogen, USA), 61.5 µl of ultrapure water and 2 µl of template DNA. The PCR temperature profile was set as described by Kittiwongwattana & Thawai (2015). The PCR product was purified using Gel/PCR purification kit (Favorgen Biotech Corp, Taiwan) and sequenced by First BASE Laboratories Sdn Bhd (Malaysia) using universal primers 27F (5'-AGAGTTTGATCMTGGCT CAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane, 1991). Pairwise alignment of the 16S rRNA gene sequences was performed, using the EzTaxon-e server (Kim et al., 2012). The 16S rRNA gene sequences were aligned with other strains obtained from GenBank/EMBL/DDBJ databases using CLUSTAL W, version 1.81 (Thompson et al., 1994). Gaps and ambiguous nucleotides were removed. The phylogenetic tree was reconstructed using the neighbour-joining method (Saitou & Nei, 1987) in the MEGA 7 software (Kumar et al., 2016). The confidence values of the branches were determined, using the bootstrap analysis based on 1,000 resamplings (Felsenstein, 1985).

3.3 Antimicrobial activities

The actinomycete strains were inoculated to 200 ml of ISP2 broth (pH 7.3) in 500 ml Erlenmeyer flask and cultured on a rotary shaker (180 rpm) at 30 °C for 14 days. The fermentation broths were partitioned with ethyl acetate twice. The ethyl acetate layers were evaporated by a rotary evaporator. Crude ethyl-acetate extracts (50 mg ml^{-1}) were used for evaluation of antimicrobial activities by disc diffusion method (Lorain, 1980). The seven tested microorganisms comprised of two gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853), four gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341, *Staphylococcus aureus* ATCC 25923, and Methicillin-resistant

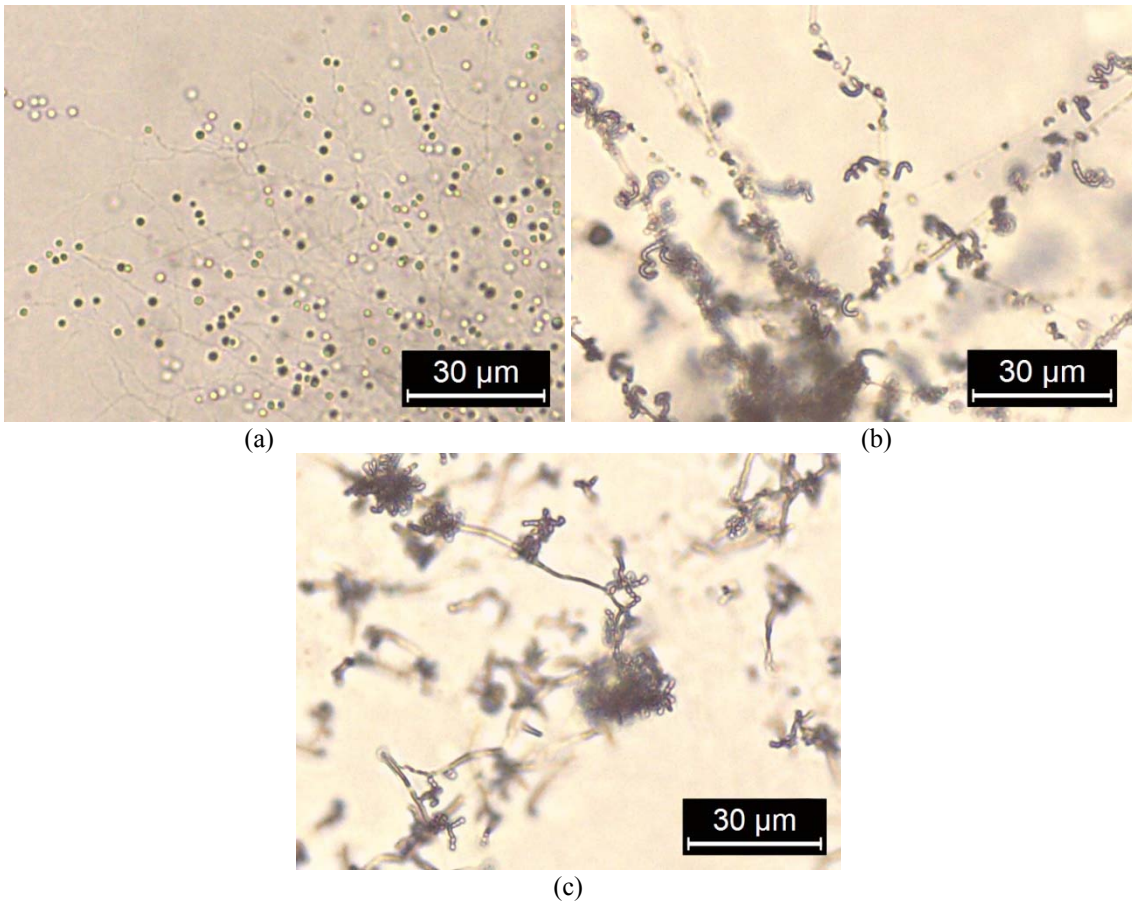
Staphylococcus aureus DMST 20654) and yeast (*Candida albicans* ATCC 10231). Gentamicin, penicillin and nystatin were used as positive controls.

4. Results and Discussion

Actinomycete strains P9, P12, and P15 were isolated from a soil sample obtained from Pho Hin Dad Waterfall, Namtok Sam Lan National Park, Saraburi province, Thailand. They were initially identified using morphological characteristics. Strain P9 produced globose bodies on branch substrate mycelium but did not produce aerial mycelium (Figure 1a). Strain P12 produced long spore chains on aerial mycelium (Figure 1b). Strain P15 produced short spore chains on aerial mycelium (Figure 1c). Cultural characteristics and phenotypic-characteristic study of each strain are described below.

Strain P9 produced pale yellowish pink to yellowish-grey substrate mycelium (Table 1). Soluble pigments were not observed in any test media. The strain grew well on ISP2 and ISP3. Moderate growth was found on ISP4, ISP7 and nutrient agar, while poor growth was observed on ISP5, ISP6, glucose asparagine agar and Czapek' sucrose agar. Growth temperatures ranged from 20 °C to 40 °C. The pH range for growth was 5-10. NaCl tolerance was found between 0-2% concentrations. Milk peptonization, gelatin liquefaction, nitrate reduction and starch hydrolysis were positive.

Strain P12 produced strong yellowish-brown to yellowish-white substrate mycelium and produced moderate yellowish-brown to white aerial mycelium (Table 1). Soluble pigments were not observed in any test media. The strain grew well on ISP2 and ISP3. Moderate growth was found on ISP4, ISP7 and nutrient agar, while poor growth was observed on ISP5, ISP6, glucose asparagine agar, and Czapek' sucrose agar. Growth temperatures ranged from 20 °C to 40 °C. The pH range for growth was 5-10. NaCl tolerance was found between 0-5% concentrations. Milk peptonization, gelatin liquefaction and starch hydrolysis were positive, but nitrate reduction was negative.



(a) (b)

(c)

Figure 1 Light micrographs of strain P9 (a), P12 (b) and P15 (c) grown on modified soil extract agar for 30 days at 30 °C, Bar 30 µm

Strain P15 produced pale-orange yellow to grayish-greeni sh yellow substrate mycelium and produced dark-grayish yellow to white aerial mycelium (Table 1). Soluble pigments were not observed in any test media. The strain grew well on ISP2 and ISP3. Moderate growth was found on ISP4, ISP7 and nutrient agar, while poor growth was observed on ISP5, ISP6, glucose asparagine agar and Czapek' sucrose agar. Growth temperatures ranged from 20 °C to 45 °C. The pH range for growth was 5-10. NaCl tolerance was found between 0-6% concentrations. Milk peptonization, gelatin liquefaction and starch hydrolysis were positive, while nitrate reduction was negative.

Table 1 Cultural characteristics of the actinomycetes strains; P9, P12, and P15

Strains	Medium	Growth	Color		
			Upper colony	Lower colony	Soluble pigment
P9	ISP2	Good	Light orange yellow	Light orange yellow	-
	ISP3	Good	Vivid orange yellow	Vivid orange yellow	-
	ISP4	Moderate	Moderate orange yellow	Moderate orange yellow	-
	ISP5	Poor	Light orange yellow	Light orange yellow	-
	ISP6	Poor	Yellowish gray	Yellowish gray	-
	ISP7	Moderate	Moderate orange yellow	Moderate orange yellow	-
	Glu.A.	Poor	Yellowish white	Yellowish white	-
	Cz.sucrose	Poor	Pale yellowish pink	Pale yellowish pink	-

Strains	Medium	Growth	Color		
			Upper colony	Lower colony	Soluble pigment
	N.A.	Moderate	Strong orange yellow	Strong orange yellow	
P12	ISP2	Good	Light grayish yellowish brown	Moderate yellowish brown	-
	ISP3	Good	White	Light yellowish brown	-
	ISP4	Moderate	Yellowish white	Grayish yellow	-
	ISP5	Poor	Moderate yellow	Moderate yellow	-
	ISP6	Poor	Grayish yellow	Grayish yellow	-
	ISP7	Moderate	Moderate yellowish brown	Moderate yellowish brown	-
	Glu.A.	Poor	Grayish yellow	Grayish yellow	-
	Cz.sucrose	Poor	Yellowish white	Yellowish white	-
	N.A.	Moderate	White	Strong yellowish brown	-
P15	ISP2	Good	White	Pale orange yellow	-
	ISP3	Good	White	Light grayish yellowish brown	-
	ISP4	Moderate	White	Pale orange yellow	-
	ISP5	Poor	Yellowish white	Yellowish white	-
	ISP6	Moderate	Dark grayish yellow	Dark grayish yellow	-
	ISP7	Moderate	Yellowish gray	Yellowish gray	-
	Glu.A.	Poor	Yellowish white	Yellowish white	-
	Cz.sucrose	Poor	White	Yellowish white	-
	N.A.	Moderate	White	Grayish greenish yellow	-

ISP2, Yeast extract-malt extract agar; ISP3, Oatmeal agar; ISP4, Inorganic salts-starch agar; ISP5, glycerol-asparagine agar; ISP6, Peptone-yeast extract iron agar; ISP7, tyrosine agar; Glu.A., Glucose asparagine agar; Cz.sucrose, Czapek' sucrose agar; N.A., Nutrient agar.

Based on their morphological characteristic, strains P9, P12 and P15 were classified to genera *Dactylosporangium*, *Nonomuraea* and *Actinomadura*, respectively. Consistently, the 16S rRNA gene sequence analysis indicated that strains P9, P12 and P15 were members of genera *Dactylosporangium*, *Nonomuraea* and *Actinomadura*, respectively. The highest similarity levels were observed between strain P9 and *Dactylosporangium sucinum* RY35-23^T (100%), strain P12 and *Nonomuraea jiangxiensis* CGMCC 4.6533^T (99.57%), and strain P15 and *Actinomadura barringtoniae* GKU 128^T (99.35%). According to the phylogenetic analysis, strain P9 was placed in the same clade of *D. sucinum* RY35-23^T with the high bootstrap value of 100%. Strain P12 was found in the same cluster containing *Nonomuraea* species, in which *N. jiangxiensis* CGMCC 4.6533^T was also found and supported by the 99% bootstrap value. Strain P15 formed a distinct clade with *Actinomadura nitritigenes* DSM 44137^T (AY035999) and *A. barringtoniae* GKU 128^T (KF667497) with the bootstrap values of 80%.

The identification of strain P9 showed 100% similarity with *Dactylosporangium sucinum* RY35-23^T (Phongsopitanun et al., 2015), which was isolated from peat swamp forest soil in Rayong province. The reason was that *Dactylosporangium* was likely a common soil inhabitant.

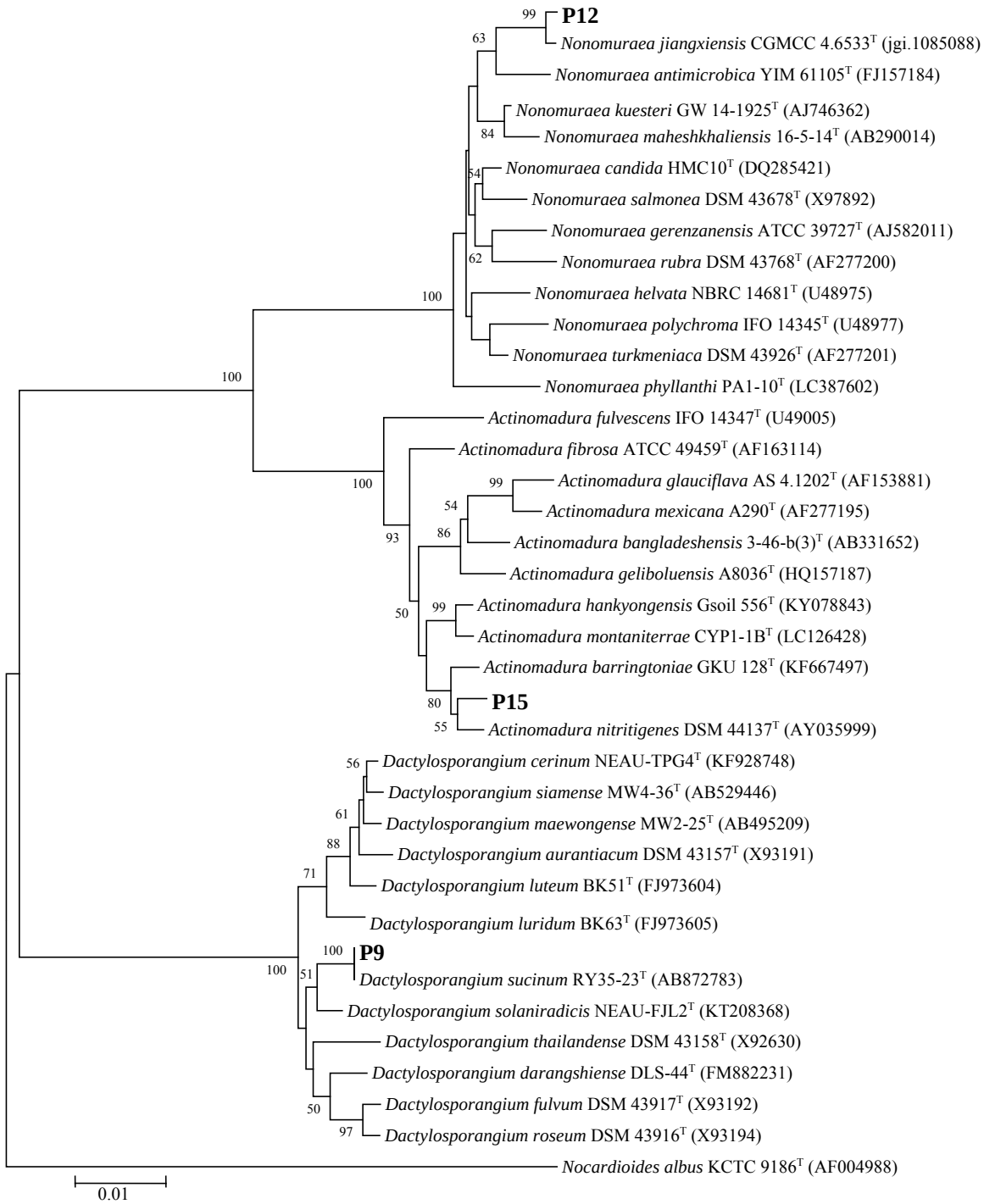


Figure 2 Neighbour-joining tree based on nearly-complete 16S rRNA gene sequences showing relationships between strain P9, P12, P15 and related species. *Nocardioides albus* KCTC 9186^T was used as an outgroup. The numbers of branches indicate the percentage bootstrap values of 1,000 replicates. Only values higher than 50% are indicated. Bar, 0.01 substitutions per nucleotide position.

For primary screening of antimicrobial activities, crude ethyl-acetate extracts obtained from these strains were tested against seven microorganisms by disc diffusion method. The results are shown in Table 2. The extracts from all strains inhibited the growth of all Gram-positive bacteria including *B. subtilis* ATCC 6633, *K. rhizophila* ATCC 9341, *S. aureus* ATCC 25923 and Methicillin-resistant *Staphylococcus aureus* DMST 20654. Additionally, the extracts from strains P9 and P15 inhibited the growth of the yeast, *C. albicans* ATCC 10231. All extracts were unable to inhibit the growth of Gram-negative bacteria, including *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853.

The antimicrobial activities from these actinomycete strains were consistent with previous studies. Antimicrobial activities were reported in twenty-four rare actinomycete strains isolated from medicinal plants of tropical rain forests collected in Xishuangbanna, China (Qin et al., 2009). The activities were against *B. subtilis* (26.1%), *S. aureus* (19.6%), *C. albicans* (17.4%) and *P. aeruginasa* (10.9%). Chaudhary et al. reported the antimicrobial activity of thirty-one actinomycete strains isolated from different soil in Sheopur district, Madhya Pradesh, India (Chaudhary et al., 2013). These actinomycetes were tested against ten Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus pyogenes* MTCC 655, *Staphylococcus saprophyticus* MTCC 6155, *Staphylococcus xylosus* MTCC 6149, Methicillin-resistant *Staphylococcus aureus* ATCC 700789, *Bacillus cereus* MTCC 430, *Bacillus subtilis* MTCC 299, Vancomycin-resistant enterococci VRE 912 and *Enterococcus faecalis* ATCC 29122) and two Gram-negative bacteria (*Shigella dysenteriae* ATCC 9754 and *Klebsiella pneumoniae* MTCC 2405). They reported that all actinomycete strains were antagonistic against at least five Gram-positive bacteria. In contrast, twenty-three actinomycete strains (74%) were against Gram-negative *K. pneumoniae* MTCC 2405, while all actinomycete strains were unable to inhibit *Shigella dysenteriae* ATCC 9754. Similarly, 42 actinomycete strains, isolated from soils of coastal islands, were reported for their high activity against Gram-positive bacteria (*B. subtilis* DSM 10, *M. luteus* DSM1790, *S. aureus* Newman, *Mycobacterium smegmatis* ATCC 700084). Moderate and low activities were observed against Gram-negative bacteria (*Chromobacterium violaceum* DSM 30191 and *E. coli* TolC, *P. aeruginosa* PA14 and *E. coli* DSM 1116) (Charousová et al., 2017). Additionally, all actinomycetes strains in this study could inhibit Methicillin-resistant *Staphylococcus aureus* DMST 20654 with a zone of inhibition approximate to other Gram-positive bacteria, which was in concordance with a previous report that found actinomycetes against Gram-positive bacteria could also inhibit Methicillin-resistant *S. aureus* (Chaudhary et al., 2013). All actinomycete strains from the present study could not inhibit Gram-negative bacteria because Gram-negative bacteria are highly resistant to many antibiotics (Lucet & Birgand, 2011).

Table 2 Antimicrobial activity of the actinomycetes strains P9, P12 and P15

Strains	Inhibition zone (mm)						
	E. coli	*P. aeruginosa*	*B.subtilis*	*K. rhizophila*	*S. aureus*	MRSA	*C. albicans*
P9	-	-	8.98	7.93	8.50	8.20	7.54
P12	-	-	12.27	12.54	7.52	13.01	-
P15	-	-	10.97	11.46	9.31	8.86	7.60

MRSA, Methicillin-resistant *Staphylococcus aureus* DMST 20654.

5. Conclusion

In the present study, three actinomycete strains, isolated from soil in Pho Hin Dad Waterfall, Namtok Sam Lan National Park, Saraburi province, Thailand, were identified as members of genera *Dactylosporangium*, *Nonomuraea* and *Actinomadura*. They showed antimicrobial activities against Gram-positive bacteria. Besides, strains P9 and P12 could inhibit yeast. Based on the result, these actinomycete strains are potential antibiotic producers. We concluded that the soil in Pho Hin Dad Waterfall was a potential source for exploring antibiotic-producing actinomycetes.

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