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Isolation and Characterisation of Actinomycetes from Mangrove forest and Hot spring Soils, Thailand

Khanungkan Klanbut [1,2*], Nutthamon Chaychoo[1], Thanapa Luchutassakul[1] and Suphaphit Phuangfueang [1]

[1]*Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok, 10520, Thailand*
[2] *Actinobacterial Research Unit, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok, 10520, Thailand*

* *Corresponding author. E-mail: khanungkan@hotmail.com*
DOI:

ABSTRACT

Fifteen isolates of actinomycetes from the mangrove forest Samet, Mueang, Chonburi and twenty four isolates from Suan Phueng hot spring, Ratchaburi were isolated, Morphology and biochemical tests were examined. Antimicrobial activity against 6 tested microorganisms; *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Kocuria rhizophila* ATCC 9341, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231 found 6 isolates which have inhibition zone on ISP2 agar by preliminary test technique. Agar disc diffusion was performed on Glucose Yeast extract agar (GYE). The strains showed greater than 99% 16S rRNA gene sequence similarity to the type strains of several recognized species of the genus Streptomyces, but in the phylogenetic tree based on 16S rRNA gene sequences of 3 isolates, it formed a distinct phyletic line and demonstrated closest relationships to *Streptomyces griseoincarnatus* LMG 19316^T (AJ781321), *Streptomyces sundarbansensis* MS1/7^T (AY550275) and *Streptomyces griseorubens* NBRC 12780^T (AB184139) with similarity percentage of 98.64, 99.79 and 99.72, respectively.

Keywords : Actinomycetes, Mangrove forest soil, Hot spring soil, Antimicrobial activity, 16s rRNA gene

INTRODUCTION

Actinomycetes are the source of outstanding bioactive compounds and extensive commercial importance (Jose and Jebakumar, 2012). The discovery of actinomycetes from numerous habitats have been continued to thrive a current poverty for new antibiotics (Lam, 2006; Poulsen *et al.*, 2011). Soil in particular produce many useful biologically active natural products, thousands antibiotics have been isolated from gram positive bacteria (Kumar *et al.*, 2014). Actinomycetes are increasingly received attention as a main producer of secondary metabolites and antibiotics (Mohseni *et al.*, 2013). Therefore isolation of new actinomycetes species is a valuable endeavor, the species belonging to the genus *Streptomyces* constitutes 50% of the total population of soil actinomycetes (Mellouli *et al.*, 2003) Actinomycetes, the gram positive soil dwelling bacteria, aerobic microorganisms with high DNA G+C contents which has the particularly geosmin odour (Miyadoh, 1993). Thus, it is considered that mangrove forest conceive to becoming a new reservoir for actinomycetes, despite of the highly productive ecosystems which comprise of unique woody plant communities and located in tropical and subtropical coastal area (Malek *et al.*, 2014). And the appealing feature from hot spring resources which are the ecology with abundant biodiversity of the organisms (Mohammad *et al.*, 2017), considerably, actinomycetes found in extreme habitats have attracted significant research interest due to their ability to produce novel products with huge commercial potential (Chaudhary and Prabhu, 2016). In Thailand, there are many mangrove forests and hot springs which are a noble site to isolate actinomycetes, according to particular chemical and physical factors contribute to the selection of species that are best adapted to that extreme environment (Dammak *et al.*, 2017). Consequently, the actinomycetes will survive under an environmental stressful, and produce several substances for multiple purposes (Jiang and Xu, 1993).

The aims of this work were to searching for a new strain of actinomycetes that can produce antimicrobial substances from mangrove forest and hot spring soils in Thailand which are the good resource to study. Here we report on isolation, taxonomic characterization of these actinomycete strains and also report on their antimicrobial activity.

MATERIAL AND METHODS

Materials

Soil sampling and pretreatment

Soil samples were collected from different habitats from mangrove forest soil Samet, Mueang, Chonburi and Suan Phueng hot spring soil, Ratchaburi. Samples were collected by inserting a shovel into the sediments 2-3 cm depth from the ground surface of each location. These samples were placed in sterile poly bags, sealed loosely, and transported immediately to the laboratory. These soil samples were air-dried for 3-5 days at room temperature, incubated at 70°C for 15 min, crushed prior to ten-fold dilution method (Chaudhary *et al*., 2013).

Isolation of Actinomycetes

Mangrove forest soil sample

Ten grams of dried soil was suspended in 90 ml sterile 0.1% tween80 and serially diluted in sterile 0.9% NaCl up to 10^{-5}. An aliquot of 0.1 ml was spread on Zhang's Starch Soil Extract agar (ZSSE) (Waksman, 1961) and Starch casein agar (SCA) with artificial seawater (18.5 g/500 ml) (Wellington and Cross, 1983) supplemented with cyclohexamide (50 µg/ml). Plates were incubated at 30°C and 45°C for 3-14 days. (Waksman *et al*., 1961 and Hopwood *et al*., 1985).

Hot spring soil sample

Ten grams of soil sample was suspended in 90 mL of 0.1% Tween80 (10^{-1}), and serially diluted in sterile 0.1% Basic Lauryl Sulfate up to 10^{-5}. An aliquot of 0.1 ml was spread on Zhang's Starch Soil Extract agar (ZSSE) (Waksman, 1961) supplemented with cyclohexamide (50 µg/ml). Plates were incubated at 45°C for 3-14 days.

The pure colonies of actinomycetes isolates were selected, isolated and maintained on International *Streptomyces* Project 2 (ISP2) medium at 30 °C and 45°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).

Characterisation of Actinomycetes

All the isolates were morphological characterised and classified by using colours of aerial mycelium on ISP2 medium by the ISCC–NBS colour system (Kelly, 1964). Biochemical characterisation were obtained from peptonisation on skimmilk agar, gelatinization on Bouillon Gelatin broth, nitrate reduction on Peptone KNO3 broth, oxidase and catalase test and starch hydrolysis on Inorganic salt-starch agar (ISP4) as described by Arai (1975)), and Williams and Cross (1971).

Antimicrobial Activity

Tested organisms

Bacillus subtilis ATCC 6633, *Escherichia coli* ATCC 25922, *Kocuria rhizophila* ATCC 9341, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231.

Determination of the antibacterial activity

The screening method of isolates consists of two steps: primary screening and secondary screening. In primary screening, the antibacterial activity of pure isolates was determined by T-streak method on Glucose Yeast extract agar (GYE). Secondary screening of isolate was done by agar disc diffusion method with crude extract of ethyl acetate after secondary metabolite extraction. Production of bioactive compound was done by submerged fermentation.

Agar disc diffusion

Actinomycetes isolates were grown in 200 ml of Yeast extract Malt extract (YEME) broth with 0.1% $CaCO_3$ in a 500-ml-capacity conical flask under sterile conditions and incubated at 30°C and 45°C for 16 days at 200 rpm rotation. After fermentation, cells were harvested through Whatman filter paper No.1 and supernatant was harvested for fermented broth. Resultant fermented broths were added to equal volume of ethyl acetate and crude extract was obtained after evaporated solvent using rotary evaporator. Harvested cells were fermented in equal volume of methanol for another 3 days and then also evaporated. The solvent phase was collected and evaporated in a desiccator. The completely dried residues were re-dissolved in methanol to be used for further studies. Antibacterial activity of partially purified

extracellular and intracellular 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 (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′AAGGAGGTGATCCAGCC3′). 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

Fifteen isolates of actinomycetes from the mangrove forest Samet, Mueang, Chonburi and twenty four isolates from Suan Phueng hot spring, Ratchaburi were isolated which pH measurement are made at a field site; 7-8. All of these strains were collected by using ZSSE and SCA medium

supplemented with cyclohexamide (50µg/ml). Cultural characteristics were performed using 14-day cultures grown at 30ºC and 45ºC on ISP2 media. The physiological and biochemical results are indicated of actinomycete strains, formed abundant, extensively branched substrate and aerial hyphae (Fig. 1) were observed directly on the agar. The colour designation of substrate mycelium and aerial mycelium was determined using the ISCC–NBS Colour Charts standard sample (Kelly, 1964); light gray, white, greenish gray, greenish white, very light green, pale yellow, moderate yellow, yellowish white, yellowish Gray, light grayish olive, light olive gray, light yellow green, grayish yellow, light brownish gray, light grayish yellowish brown on ISP2 agar. Biochemical characterisation were obtained both of positive and negative results from peptonisation on skimmilk agar, gelatinization on Bouillon Gelatin broth, nitrate reduction on Peptone KNO3 broth, oxidase and catalase test and starch hydrolysis on Inorganic salt-starch agar (ISP4).

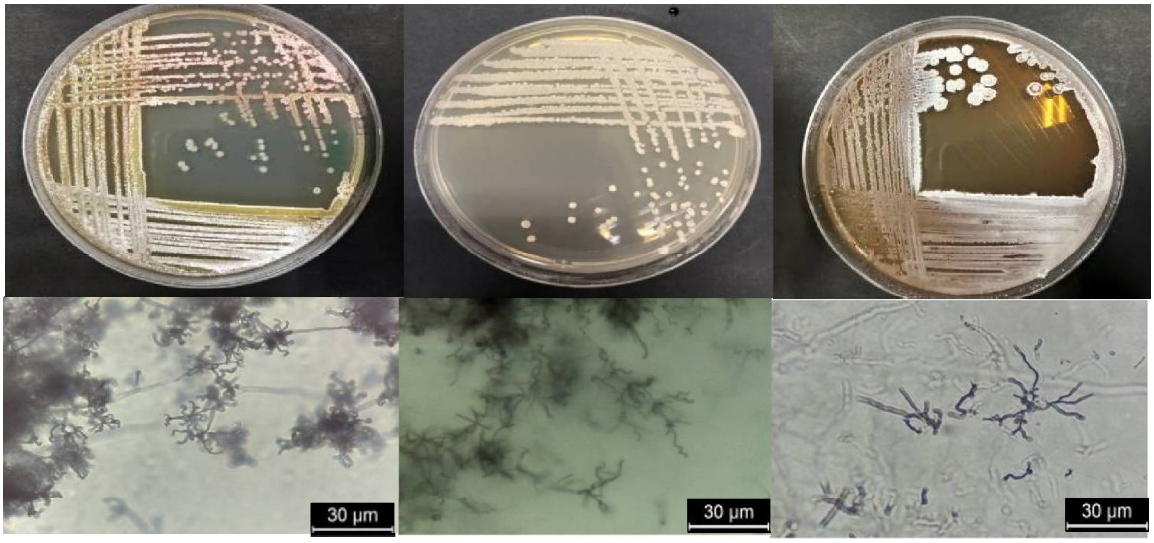

Figure 1. Morphological appearance colonies on ISP2 agar medium and spore arrangement of some actinomycetes.

Antimicrobial activity

Preliminary screening for antimicrobial activity of 39 isolates were tested against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC9341, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231

using T-streak method, found 6 isolates which have inhibition zone. 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.

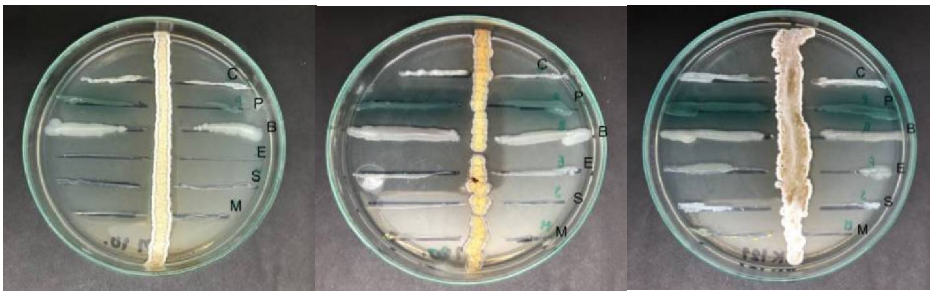

Figure 2. Some of actinomycetes against tested microorganisms using T'streak.

Unfortunately, the antimicrobial activity of partially purified extracellular and intracellular crude extracts could not be found on agar disc diffusion test. Considerably, due to solubilizing agents or solvents were not suitable, for further study the polarity of extraction solvents should be more concerned.

Molecular identification

The 16S rRNA gene sequence and blast analyses confirmed that the active isolates belonging to the genus *Streptomyces* that had percent similarity 98. 64- 99. 79 and sequences were submitted to GenBank with accession number as showed as Table 1.

Table 1. Identification of nucleotide sequence using 16s rRNA gene.

Isolate No.	Accession number	Identification	%similarity (Total nt)
BK530	MS1/7	*Streptomyces sundarbansensis*	99.79 (1,537)
BK830	NBRC 12780	*Streptomyces griseorubens*	99.72 (1,468)
SM1322211	LMG 19316	*Streptomyces griseoincarnatus*	98.64 (1,427)

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 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1376 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016)

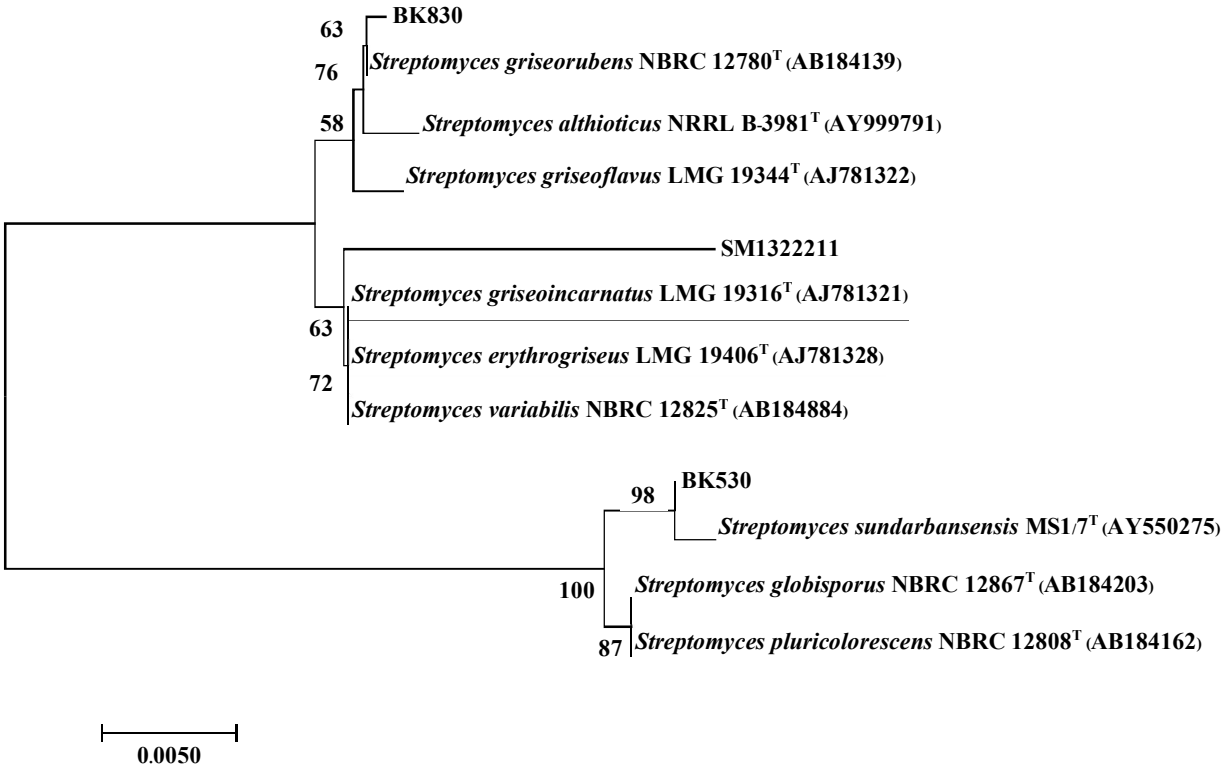

Figure 3. Phylogenetic tree based on 16S rRNA gene sequences using neighbour-joining method for 4 isolates of *Streptomyces* spp. and their closely related type strains.

DISCUSSION

Thirty-nine actinomycete strains were isolated from Thai hot spring sediments and from the mangrove forest Samet, Mueang, Chonburi. These strains were grouped using morphological characteristic, physiological, biochemical properties. The generic identities of these selected actinomycete isolate in each group were determined by using a procedure that combined morphological, chemotaxonomic and 16S rRNA gene sequence- based phylogenetic analyses.

The actinomycete from Suan Phueng hot spring, Ratchaburi consisted of fifteen strains, the colours of the aerial mycelium were light gray, white, greenish gray, greenish white, very light green, pale yellow, moderate yellow and yellowish white. The morphological characteristics of these isolates were consistent with their classification in the genus *Streptomyces*. The representative strain, SM1322211, was most closely associated with *Streptomyces griseoincarnatus* LMG 19316^T (AJ781321) (Lanoot *et al*., 2005) in the neighbor-joining analysis (Fig. 3) and shared the highest 16S rRNA gene sequence similarity percentage of 98.64 with *Streptomyces griseoincarnatus* LMG 19316^T (AJ781321).

The actinomycete from the mangrove forest Samet, Mueang, Chonburi consisted of twenty four strains, the colours of the aerial mycelium were yellowish white, yellowish Gray, light grayish olive, light olive gray, light yellow green, grayish yellow, light brownish gray and light grayish yellowish brown. The morphological characteristics of these isolates were consistent with their classification in the genus *Streptomyces*. The representative strains; BK530, was most closely associated with *Streptomyces sundarbansensis* MS1/7^T (AY550275) (Arumugam *et al*., 2011) in the neighbor-joining analysis (Fig. 3) and shared the highest 16S rRNA gene sequence similarity percentage of 99.79 with *Streptomyces sundarbansensis* MS1/7^T (AY550275). Strain BK830, was most closely associated with *Streptomyces griseorubens* NBRC 12780^T (AB184139) (Prasad *et al*., 2013) in the neighbor-joining analysis (Fig. 3) and shared the highest 16S rRNA gene sequence similarity percentage of 99.72 with *Streptomyces griseorubens* NBRC 12780^T (AB184139).

CONCLUSION

In this study, we successfully isolated the actinomycetes from the sediments collected from hot spring pond located in Suan Phueng, Ratchaburi province and from the mangrove forest Samet, Mueang, Chonburi. These actinomycete strains were identified using the morphological property and 16S rRNA gene sequence analysis. They belonged to the member of genera Streptomyces; BK530 has 99.79% similarity to *Streptomyces sundarbansensis*, BK830 has 99.72% similarity to *Streptomyces griseorubens*, SM1322211 has 98.64% similarity to *Streptomyces griseoincarnatus*, which isolates SM1322211 be inclined to a new species. The crude ethyl acetate extract from the fermentation broth could not found the anti-bacterial activity This result implied that the hot spring sediments are a great source for discovery of new actinomycetes.

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