Filamentous Fungi PKF121 Isolated from Dry Dipterocarp Forest Soil in Northeast Thailand Produces Antimicrobial Agents Active against Methicillin-Resistant *Staphylococcus aureus*

Phimpha Khowangklang and Nawarat Nantapong*

***Abstract*—** An antimicrobial-producing fungi PKF121 strain was isolated from dry dipterocarp forest soil in Suranaree University of Technology, Nakhon Ratchasima, Thailand. Morphological characteristics of PKF121 showed grayish green color, granular powdery colony and septate hyphae which indicated the genus *Penicillium*. The species level of PKF121 was determined by the internal transcribed spacer (ITS) sequence analysis. The results of morphological characteristics and ITS sequence analysis, thus, concluded that PKF121 could be classified as *P. citrinum*. Antimicrobial activity analysis showed that PKF121 was active against *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Candida albicans*, *Saccharomyces cerevisiae* as well as drug-resistant strain, methicillin-resistant *Staphylococcus aureus* (MRSA).

***Keywords*—**Antimicrobial, *Penicillium*, Methicillin-resistant *Staphylococcus aureus*, MRSA.

I. Introduction

Infectious diseases caused by pathogenic microorganisms including drug-resistant bacteria have been the leading cause of illness and death in human [1]. The major type of drug-resistant bacterial strains causing infectious diseases include methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Staphylococcus aureus* (VRSA), vancomycin-resistant *Enterococcus* (VRE), penicillin-resistant *Streptococcus pneumonia* (PRSP) and multidrug-resistant *Clostridium difficile* (MDR) [2]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of hospital-acquired infection. It leads to a higher medical cost, prolonged hospital stay and increased mortality [3]. Thus, there is an increasing need of new and effective drugs for the treatment of infectious diseases. One potential source of novel antibiotics is microorganisms. Many previous studies have shown that antimicrobial-producing microorganisms are widely distributed in natural habitats, especially in soil [4-8]. Soil microorganisms are an important source for bioactive secondary metabolites such as antimicrobial drugs, anticancer drugs, insecticides and herbicides [9, 10]. Soil microorganisms that are commonly found to produce antibiotics include actinomycetes (70%), fungi (20%) and eubacteria (10%) [11]. In comparison to other natural sources, microorganisms is highly diverse but narrowly explored. The study based on the estimation of microbial populations has showed that only 1% of bacteria and 5% of fungi have been classified. The rest remain unexplored for their antimicrobial activity [12].

Soil fungi play an important role within the soil in relation to nutrient cycling and disease suppression [13, 14]. Since the 1940s, fungi have been used for the production of antibiotics [11]. Antimicrobial drugs produced by fungi include penicillin, cephalosporin, griseofulvin, fumagillin and fusidic acid [15-17]. Several methods were used for classification and identification of fungi which included colony morphology, cell morphology and ITS sequence analysis. However, the combination of macroscopic characteristics, microscopic characteristics and sequence analysis of ITS enabled an identification of fungi to the genus-species level [18, 19].

It has been reported that 32.1% of Thailand is covered by various forest habitats [20]. Although, the study of microbial diversity in Thailand have been conducted, an investigation of soil microorganisms including fungi from many part of Thailand remain unexplored. Thus, the present study was focused on the isolation and identification of antimicrobial-producing fungi from forest soil in Nakhon Ratchasima province, Thailand where the study of antimicrobial-producing fungi has never been reported.

II. Materials and Methods

A. Sample Collection

Soil Samples were collected from different area in Suranaree University of Technology, Nakhon Ratchasima, Thailand. Soil samples were randomly taken at 10-15 cm depth from surface. The soil were kept in polypropylene bags and transferred to the laboratory in icebox.

[1]Phimpha Khowangklang and Nawarat Nantapong* are with the School of Preclinical Science, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand

B. Media and Culture Conditions

Potato dextrose broth (PDB), potato dextrose agar (PDA) and Muller-Hinton agar (MHA) were purchased from Hi-Media, India. Sabouraud dextrose agar (SDA) was prepared by dissolving 10 g of peptone, 40 g of glucose and 15 g of agar in 1 L of water and adjusted a pH to 5.6. PDA and PDB medium were used for isolation and cultivation of fungi. Cultivation temperature of fungal strain was 28 ^{o}C. MHA and SDA were used for the determination of antimicrobial activity. The incubation temperature for antimicrobial activity test was 37 ^{o}C.

C. Strain of Test Pathogens

The pathogenic strains used in this study were purchased from Department of Medical Sciences Thailand (DMST) and Thailand Institute of Scientific and Technological Research (TISTR). They were *Staphylococcus aureus* TISTR1466, methicillin-resistant *Staphylococcus aureus* DMST20654 (MRSA), *Bacillus subtilis* TISTR008, *Bacillus cereus* TISTR687, *Candida albicans* TISTR5779, *Candida tropicalis* TISTR5174 and *Saccharomyces cerevisiae* TISTR5049.

D. Isolation of Fungi from Soil Sample

One gram of soil samples was suspended in Erlenmeyer flask containing 99 ml sterile water and incubated at room temperature with shaking condition for 30 min. Soil suspension was serially diluted and spreaded onto PDA plates supplemented with 50 mg/l chloramphenicol. The plates were incubated at room temperature for 7-14 days. After incubation, the suspected fungal colonies were sub-cultured to PDA plates without antibiotics and used for further study.

E. Determination of Antimicrobial Activity

The antimicrobial activity of soil isolates were determined by cross-streak method. The fungal isolate was inoculated on MHA or SDA by streaking at one side of a petri dish. MHA medium was used for bacterial sensitivity test while SDA medium was used for yeast sensitivity test. The plates were incubated at 28^{o}C for 5 days to allow the organisms to produce and release antimicrobial substance into the agar. After incubation, the test pathogens were streaked perpendicularly to the line of fungal colonies and incubated at 37^{o}C for 24-48 h. The zone of inhibition in millimeter against pathogenic strains was measured.

F. The Internal Transcribed Spacer (ITS) Region Sequencing and Sequence Analysis

Genomics DNA of fungal strain was isolated from cell grown in 10 ml PDB at 28^{o}C with 200 rpm shaking condition for 5 days. Extraction of fungal genomic DNA was performed as described by Al-Samarrai & Schmid (2000) [21]. The fungal genomic DNA was used as DNA template for PCR amplification of ITS region. The PCR amplification of ITS region was performed by using universal primer, ITS5 (5' – GGAAGTAAAAGTCGTAACAAGG – 3') and ITS4 (5' – TCCTCCGCTTATTGATATGC – 3') [22]. The thermal cycling conditions were as follows: initial denaturation at 95^{o}C for 5 min followed by 30 cycles of denaturation at 95^{o}C for 30 sec, annealing at 50^{o}C for 1 min, extension at 72^{o}C for 1 min and final extension at 72^{o}C for 7 min. The amplified fragments were purified using NucleoSpin® Gel and PCR clean-up kit (MACHEREY-NAGEL, Germany). The purified PCR product was submitted for DNA sequencing at Macrogen Inc., Korea. The sequence of ITS region was compared with known sequence from NCBI GenBank, database.

III. Results and Discussion

Natural compounds from soil microorganisms are the most important source for the production of bioactive agents used in pharmacy, industry and agriculture [23]. Antimicrobial metabolites play an important role in the treatment of bacterial and fungal infectious diseases [24]. The emergence of antibiotic-resistant bacteria decreases the efficacy of therapeutic drugs [25]. Therefore, it is necessary for the search of the novel effective antibiotics. In this study, we attempted to isolate the antimicrobial-producing fungi from forest soil in Suranaree University of Technology. This area is covered with dry dipterocarp forest. Dry dipterocarp forest soil is less water retention, sandy loam or gravel and low nutrients which could establish slightly extreme condition. Microorganisms live under an extreme condition usually produce the secondary metabolites such as antibiotics and other defensive compounds for their survival [26]. It has been shown that soil from dry dipterocarp forest in Suranaree University of Technology in northeast of Thailand contains a variety of antibiotic-producing actinomycetes [27]. Therefore, the screening and isolation of fungal strain from this area might lead to the discovery of antibiotic drugs to combat pathogenic organisms especially, drug-resistant strain.

A. Isolation and Classification of Soil Fungi PKF121

In this study we obtained antimicrobial-producing fungal strain, PKF121 from forest soil in Suranaree University of Technology, Thailand. This strain showed antimicrobial activity against test pathogens which were Gram-positive bacteria and yeasts. The identification of PKF121 was based on colony morphology, cell morphology and ITS sequence. The colony of PKF121 appeared grayish green color with a white periphery, granular powdery colony and the reverse side showed pale to yellowish in color on PDA medium (Fig. 1A-1B). PKF121 showed septate hyphae with globose to sub-globose conidia and philides flask shaped (Fig. 1C). From these results, it could be suggested that PKF121 might belong to the genus *Penicillium*. The macroscopic and microscopic characteristics of PKF121 are summarized in Table 1.

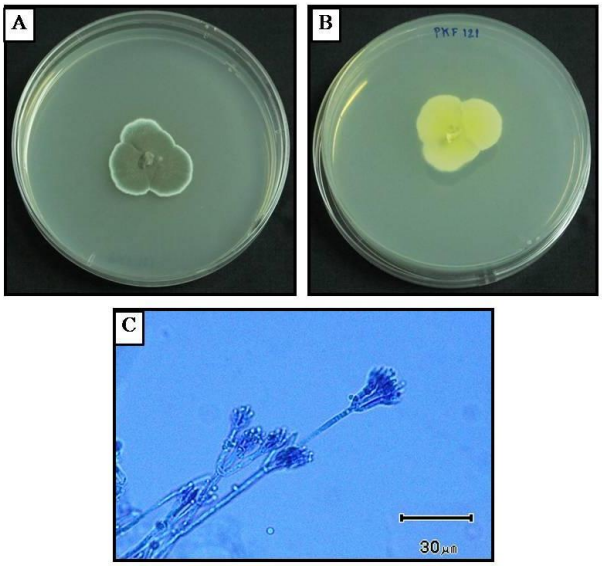

Fig. 1: The colony morphology of fungal isolate PKF121 on PDA medium: (A) obverse; (B) reverse. Cell morphology of PKF121 under light microscope (C)

TABLE 1:
MACROSCOPIC AND MICROSCOPIC MORPHOLOGIES OF PKF121

Characteristics	Observation
Surface texture	Velutinous
Colony growth appear	Radially sulcate
Color of aerial mycelium	Greyish-turquoise with a white periphery
Color of the reverse	Pale yellow
Hyphae	Septate hyphae, Smooth-walled
Phialides	Flask-shaped
Conidia	Globose to sub-globose

The internal transcribed spacer (ITS) sequence analysis was used for the identification of PKF121 in the species level. To amplify the ITS region of PKF121, ITS5 and ITS4 primers were used. The sequence of ITS region was blasted and aligned with known species from NCBI GenBank database. The blast result of PKF121 revealed 99% similarity to *Penicillium citrinum* strain IFM63148 (Fig. 2). Thus, this strain, could be classified as *Penicillium citrinum*.

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PKF121     1   GTGTTGCCCGAACCTATGTTGCCTCGGCGGGCCCCGCGCCCGCCGACGGCCCCCCTGAAC  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
IFM63148  69   GTGTTGCCCGAACCTATGTTGCCTCGGCGGGCCCCGCGCCCGCCGACGGCCCCCCTGAAC  128

PKF121    61   GCTGTCTGAAGTTGCAGTCTGAGACCTATAACGAAATTAGTTAAAACTTTCAACAACGGA  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
IFM63148  129  GCTGTCTGAAGTTGCAGTCTGAGACCTATAACGAAATTAGTTAAAACTTTCAACAACGGA  188

PKF121    121  TCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCA  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
IFM63148  189  TCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCA  248

PKF121    181  GAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGC  240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
IFM63148  249  GAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGC  308

PKF121    241  ATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCCCGTcccc  300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
IFM63148  309  ATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCCCGTCCCC  368

PKF121    301  cccGCCGGGGGGGACGGGCCCGAAAGGCAGCGGCGGCGCCGCGTCCGGTCCTCGAGCGTAT  360
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
IFM63148  369  CCCGCCGGGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTAT  428

PKF121    361  GGGGCTTCGTCACCCGCTCTAGTAGGCCCGGCCGGCGCCAGCCGACCCCCAACCTTTAAT  420
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
IFM63148  429  GGGGCTTCGTCACCCGCTCTAGTAGGCCCGGCCGGCGCCAGCCGACCCCCAACCTTTAAT  488

PKF121    421  TATCTCAGGTTGACCTCGGATCAGGTAGGGATA  453
               |||||||||||||||||||||||||||||||
IFM63148  489  TATCTCAGGTTGACCTCGGATCAGGTAGGGATA  521
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Fig. 2: Alignment sequence between ITS sequence of PKF121 and *Penicillium citrinum* IFM63148

B. Determination of Antimicrobial Activity of PKF121

The determination of antimicrobial activity of PKF121 was done by cross-streak method (Fig. 3). The pathogenic strains used in this study were *Staphylococcus aureus* TISTR1466, methicillin-resistant *Staphylococcus aureus* DMST20654 (MRSA), *Bacillus subtilis* TISTR008, *Bacillus cereus* TISTR687, *Candida albicans* TISTR5779, *Candida tropicalis* TISTR5174 and *Saccharomyces cerevisiae* TISTR5049. The antimicrobial activities of fungal strain PKF121 against test pathogens are shown in Table 2. The results indicated that PKF121 exhibited antibacterial activity toward methicillin-resistant *S. aureus*, *S. aureus*, *B. subtilis* and *B. cereus*. The strain PKF121 showed the highest activity against *B. subtilis* (42 mm.) followed by *B. cereus* (35 mm.), MRSA (29 mm.) and *S. aureus* (22 mm.). The PKF121 also showed antiyeast activity against *C. albicans* (17 mm.) and *S. cerevisiae* (8 mm.). Antimicrobial agent produced from PKF121, however, was not active against *C. tropicalis.*

In Thailand, the strains of antimicrobial-producing fungi were isolated from soil collected in Chiang Mai, Khon Kaen, Bangkok and Nakhon Si Thammarat. They were *Neosartoya hiratsukae, Neosartorya pseudofischeri, Neosartorya spinosa, Lasiodiplodia theobromae, Sclerotium rolfsii, Phytophthora palmivora, Colletotrichum capsici, Pyricularia grisea, Alternaria sp., Helminthosporium maydis, Rhizoctonia*

solani, *Fusarium oxysporum*, *Colletotrichum gloeosporioides*, *Trichoderma harzianum*, *Aspergillus flavus*, *Trichoderma brevicompactum*, *Trichoderma atroviride*, *Fusarium solani* and *Penicillium sp*. [28-31]. However, *P. citrinum* with antimicrobial and antiyeast activities was reported from Surat Thani province in southern part of Thailand [32]. To our best knowledge, this study provides the first report of antimicrobial producing *P. citrinum* isolate from soil in northeast of Thailand. It should be noted that PKF121 exhibited a relatively high antibacterial activity against MRSA. The study of PKF121 may lead to the development of antimicrobial drug to treat drug-resistant pathogenic strains. Thus, dry dipterocarp forest soil in Suranaree University of Technology has proven to be an attractive source for the search of antimicrobial substances.

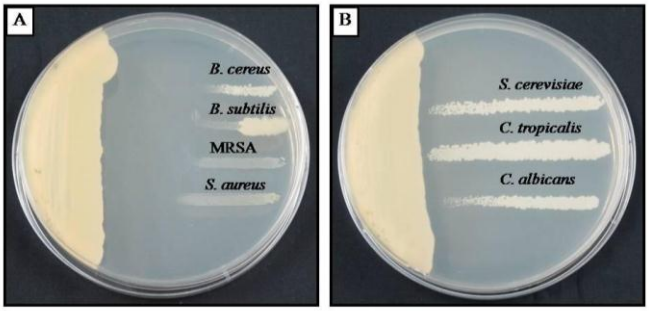

Fig. 3: Antimicrobial activity of fungal strain PKF121 against pathogenic bacteria (A) and yeasts (B) by cross-streak method

TABLE II:
ANTIMICROBIAL ACTIVITY OF PKF121 AGAINST TEST PATHOGENS BY CROSS-STREAK METHOD

Test pathogens	Zone of inhibition (mm)
Gram-positive bacteria	
Staphylococcus aureus TISTR1466	22
Staphylococcus aureus DMST20654 (MRSA)	29
Bacillus subtilis TISTR008	42
Bacillus cereus TISTR687	35
Yeasts	
Candida albicans TISTR5779	15
Candida tropicalis TISTR5174	0
Saccharomyces cerevisiae TISTR5049	8

IV. CONCLUSION

Fungal strain PKF121 was successfully isolated from dry dipterocarp forest soil in Suranaree University of Technology, Thailand. This strain was classified as *Penicillium citrinum*. It is active against test pathogens including methicillin-resistant *Staphylococcus aureus*. The study of this strain might be further use for the treatment of MRSA infections.

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