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SHORT COMMUNICATION

Application of Phenol Pretreatment for the Isolation of Rare *Actinomycetes* from Indonesian Soil

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Phenol treatment was applied for isolation of rare *Actinomycetes* using 25 soil samples collected from Pulau Seribu, Tanjung Redep, Manokwari, and Halmahera. The samples were air-dried and suspended in 1.5 % (w/v) phenol solution at 30 °C for 30 minutes, and subsequently cultured on plates of humic acid-vitamin agar (HVA) medium supplemented with cycloheximide (50 µg mL^{-1}) and nystatin (50 µg mL^{-1}). A total of 61 isolates were obtained and the most dominant isolates were not *Streptomyces* (only 24.6%), whereas other genera such as *Micromonospora*, *Actinomadura*, *Microbispora* and *Polymorphospora* were isolated with ratios of 49.2%, 13.1%, 9.8%, and 3.3%, respectively.

Key words: phenol method, rare-*Actinomycetes*, selective isolation method, soil sample

Perlakuan phenol diterapkan untuk mengisolasi *Actinomycetes* selain dari *Streptomyces* dari 25 sampel tanah yang diperoleh dari Pulau Seribu, Tanjung Redep, Manokwari, dan Halmahera. Sampel dikering anginkan dan disuspensi dalam 1,5% larutan fenol pada suhu 30 °C selama 30 menit, kemudian dikultur pada media asam humat-vitamin agar (HVA) yang dilengkapi dengan cycloheximide (50 µg mL^{-1}) dan nistatin (50 µg mL^{-1}). Sebanyak 61 isolat diperoleh dengan komposisi: *Streptomyces* hanya 24,6%, sedangkan marga lain seperti *Micromonospora*, *Actinomadura*, *Microbispora* dan *Polymorphospora* diperoleh masing-masing dengan rasio 49,2%, 13,1%, 9,8%, dan 3,3%.

Kata kunci: *Actinomycetes*, metode isolasi selektif, perlakuan fenol, sampel tanah

Actinomycetes (Actinobacteria) is a group of prokaryotic organisms belonging to the subdivision of the Gram-positive bacteria. Most of them are in the subclass *Actinobacteridae* and order *Actinomycetales*. All members of this order are characterized in part by the high G+C content (>55 mol%) in their DNA (Stackebrandt *et al.* 1997). *Actinomycetes* are a group of microbes, of which many of the members can produce antibiotics and antitumors (Chantongcome *et al.* 2009). A total of 7899 bioactive compounds have been identified up to 1988; 67% from *Actinomycetes*, 12% from bacteria, and 20% from fungi. Today, known bioactive metabolites are more than 22 000, 10 100 of which are produced by *Actinomycetes*. So far, 140 to 160 antibiotics have been used in human therapy and agriculture, and 100 to 120 of them are produced by *Actinomycetes* (Berdy 2005).

New antimicrobial agents are desperately needed to combat the increasing number of antibiotic resistant pathogenic microbial strains. Natural products remain the most propitious source of novel antibiotics. It is widely accepted that *Actinomycetes* are prolific producers of natural bioactive compounds. The efficiency of research to discover new compounds with novel chemical structures can be increased with intensive efforts in isolating and screening rare genera of microorganisms (Takahashi, 2004 ; Kumar *et al.* 2010). Rare *Actinomycetes* are usually regarded as strains whose isolation frequency much lower than those strains isolated by conventional methods. Past and present efforts in the isolation of rare *Actinomycetes* have discovered some genera, such as *Actinomadura*, *Actinoplanes*, *Micromonospora*, *Microtetraspora*, that had been recovered from many soil samples (Lazzarini *et al*. 2001; Hayakawa *et al.* 2010). Although researchers have utilized numerous approaches to isolate new microbes, the number of microorganisms that have been successfully grown represents only a small portion of the total that exists (Khana *et al.* 2011). Hawksworth (1997) reported that the number of known bacteria comprises not more than 10% of the estimated total number of species in the world. The isolation of unknown microbial strains thus

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represents an area of great potential.

Indonesia is known as a country with megadiversity of microorgaisms, *i.e.* it lies geographically on a tropical region with the highest level of biodiversity that it has high potential as a source of novel microbial bioactive metabolites. Efforts to isolate and evaluate microbial bioactive compounds from Indonesia, especially from *Actinomycetes*, have expanded significantly in recent years. However, most of the isolates recovered on agar plates have been identified as genus *Streptomyces*, which are the dominant *Actinomycetes* in soil. Lisdiyanti (2006) reported, a total of 982 *Actinomycetes* isolated from Indonesia in 2003 and 2004 using three isolation methods including sodium dodecyl sulfate yeast extract (SDS-YE), rehydration-centrifugation, and dry heating. Her study revealed that on the basis of 16S rRNA, 66.1% isolates were identified as *Streptomyces* and 33.8% isolates were Non-*Streptomyces* Groups.

For the purpose of screening rare bioactive molecules, the adoption of improved methodologies for isolating the uncommon and less studied, rare *Actinomycetes* is required to avoid re-isolating strains that produce known bioactive metabolites and to improve the quality of the natural products screened (Takahashi and Omura 2003; Berdy, 2005; Khana *et al.* 2011). Over the past year, we have attempted various methods to isolate novel actinomycetes, using three isolation methods including wet method, acid treatment, dry heating on the basis of 16S rRNA, 79.5% isolates were identified as *Streptomyces* and 20.5% isolates are as Non-*Streptomyces* groups.

In this paper we focused on the application of phenol pretreatment for isolating rare *Actinomycetes* from Indonesian soil sample. This method selectively isolated *Streptomyces violaceusniger* cluster (Hayakawa *et al*. 2004), *Micromonospora* (Hayakawa *et al*. 1991), *Dactylosporangium*, *Microbispora* (Takahashi and Omura, 2003), and *Actinomadura* (Hayakawa *et al*.1995) from Japan soil sample.

A total of 25 soil samples were collected from Pulau Seribu (West Java), Tanjung Redeb (East Kalimantan), Manokwari (Papua), and Halmahera (North Maluku). Samples were passed through a 2 mm mesh sieve and air-dried at room temperature for 1 week.

One gram air-dried sample were mixed with 10 mL sterilized water and vigorously stirred for 2 min. After allowing the tube to stand for 1 min, 1 mL of supernatant was transferred to 9 mL of 1.5% phenol solution. Then the mixture was maintained at 30 °C with occasional stirring. After incubation for 30 min, aliquots of mixture were spread onto humic acid-vitamin agar supplemented with cycloheximide (50 μg mL^{-1}) and nystatin (50 μg mL^{-1}). The experiments were done in replicates and then all plates are incubated at 28 °C for 1 to 3 weeks.

A total of 61 actinomycete isolates were successfully isolated from 25 soil samples. Twenty seven isolates were isolated from Halmahera, 21 isolates from Manokwari, 8 isolates from Pulau seribu, and 5 isolates from Tanjung redep (Table 1). Colonies having characteristic features such as powdery appearance with convex, concave or flat surface and color ranging from white, gray to pinkish and yellowish were selected. Colonies observed on the first and second days were eliminated because *Actinomycetes* were considered as slow grower (Currie 2006).

Rare *Actinomycetes* have usually been regarded as genera of *Actinomycetes* whose isolation frequency by conventional methods is much lower than that of *Streptomyces*. Consequently, basic knowledge on the habitat, physiology and productivity of molecules of rare *Actinomycetes* develops rather slowly. Due to the discovery of *Actinomycetes* with ecologically significant properties the screening extends into uncommon environments. For the purpose of screening novel bioactive molecules, several factors must be considered: pretreatment, selective medium and recognition of candidate colonies on a primary isolation plate.

Production of spore mass was detected on the 21-day old cultures on Humic Vitamin Agar. Spore ornamentation was observed by Olympus BX51 microscope. The DNA was isolated using InstaGene matrix kit for DNA isolation. The pellet was lysed using a lysing matrix, combined with 100μL, and centrifugation for 3 min at 15 000 x g.

Identification using the 16S rDNA was conducted by PCR using a 9F forward primer, GAGTTTGAT (C/T)(C/A)TGGCTCAG; and a 1541R reverse primer, AAGGAGGTG(A/T)TCCA(A/G)CC. The reaction mixture (50 μL total volume) contained 30 μL ddH2O, 5 μL of MgCl2, 5 μL of 10X Buffer 4 μL of deoxynucleoside triphosphates, 1 μL of each primer, 0.25 μL of Takara Taq Polymerase (Takara Bio Inc, Japan), and 5 μL of cell lysis as the template. PCR conditions were as follows: denaturation at 98 °C for 20 s, annealing at 52 °C for 45 s, and elongation at 72 °C for 2 min. A total of 30 cycles were performed, followed by a final elongation for 4 min at 72 °C. PCR products were purified with a Geneaid PCR Fragments Extraction Kit according to the manufacturer's

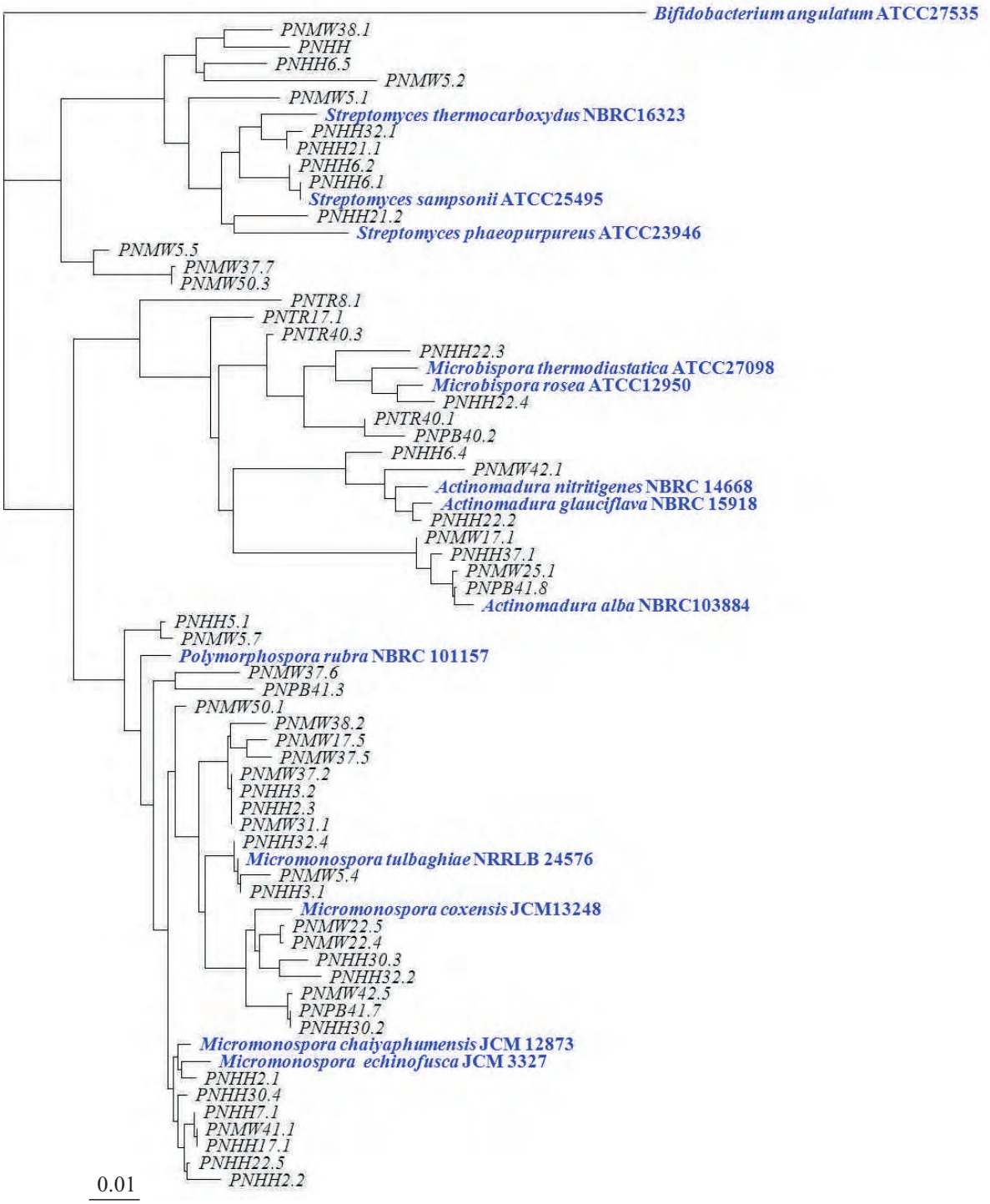

Fig 1 Phylogenetic trees showing the relationship between the isolates and related microorganism in the order *Actinomycetales* based on 16S rRNA sequences. Bar : 1% estimated sequence divergence.

instructions (Geneaid, Taiwan). Amplicon was sequenced with an automatic sequence analyzer (Applied Biosystems 3130 DNA Analyzer; Applied Biosystems, CA, USA) using the BigDye_ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Related sequences were identified by performing sequence database searches using BLAST. Sequence data for related species were retrieved from GenBank. Phylogenetic analysis was performed using CLUSTAL W software.

Phylogenetic analysis by 16S rDNA sequencing (1500 bp) showed a various similarity between the strains (Fig 1). Table 1 shows that the most frequent genus found was *Micromonospora* sp. (30 isolates) followed by *Streptomyces* sp. (15 isolates), *Actinomadura* sp. (8 isolates), *Microbispora* sp. (6 isolates), and *Polymorphospora* sp. (2 isolates).

Analysis of sequence of 16S rDNA from the 61

Table 1 Correlation between sampling place and isolated *Actinomycetes*

Sampling place	Number of strain	*Streptomyces*	*Micromonospora*	*Actinomadura*	*Microbispora*	*Polymorphospora*
Pulau Seribu	8	2	5	1	0	0
Tanjung redeb	5	0	0	1	4	0
Manokwari	21	6	11	3	0	1
Halmahera	27	7	14	3	2	1
Total	61	15	30	8	6	2

Table 2 Correlation number between isolated *Actinomycetes* and their homologies from database

Genus	Number of strain	<98%	98-99%	99-100%	100%
Streptomyces	15	3	4	6	2
Micromonospora	30	0	9	19	2
Actinomadura	8	1	1	5	1
Microbispora	6	1	1	4	0
Polymorphospora	2	0	1	1	0
Total	61	5	16	35	5

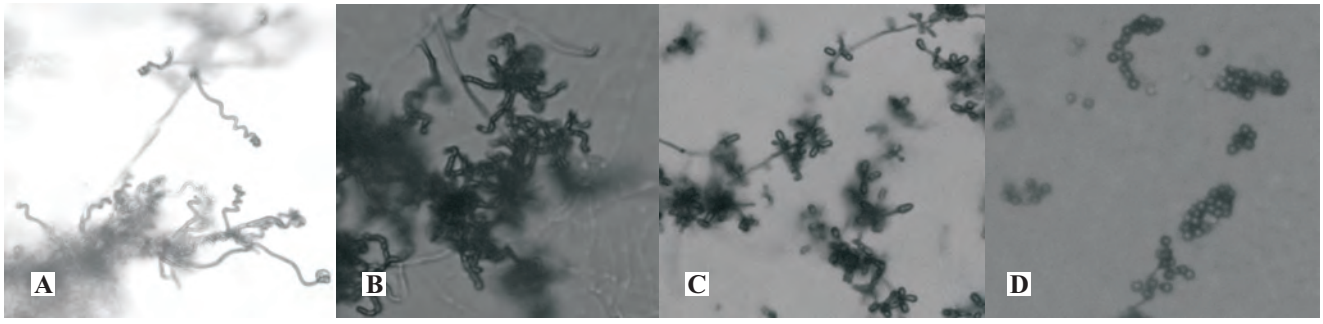

Fig 2 Cell morphology of isolated *Actinomycetes* (100X). (A) *Streptomyces* sp.; (B) *Actinomadura* sp.; (C) *Microbispora* sp.; (D) *Micromonospora* sp.

isolated *Actynomycetes* strain indicated that a total 5 strains were novel *Actinomycetes* strains exhibiting low similarity (<98%) with their close relatives (Table 2). To compare and confirm the identification by genetic analysis, these isolated *Actynomycetes* strains were identified by morphological examination. The colonies were categorized by microscope observation. From a total of 61 isolates of *Actinomycete*s, 15 isolates were identified as *Streptomyces* (spore chains with coiling, spiral and looped) (Fig 2A), 8 isolates *Actinomadura* (spore chains were straight and open hooked) (Fig 2B), 6 isolates *Microbispora* (longitudinal pair of spores on aerial mycelium) (Fig 2C), and 30 isolates *Micromonospora* (clusters of single conidia on substrate mycelium) (Fig 2D).

When the soil samples were cultured without pretreatment, the most of the colonies recovered were bacteria other than *Actinomycetes*, followed by *Streptomyces*, fungi, and non-*Streptomyces* *Actinomycetes*. When the soil was air-dried, the number of other bacteria decreased, whereas the number of *Streptomyces* colonies increased. Over the past year, we applied various methods to isolate rare *Actinomycetes*, such as: (1) Wet Method (*Streptomyces* 65%, *Nocardia* 29%, *Amycolatopsis* 3.4%, *Kitasatospora* 1.6%, and *Micromonospora* 0.2%.), (2) Acid Treatment (*Streptomyces* 89%, *Kitasatospora* 5.5%, *Nocardia* 4.5 %, *Amycolatopsis* 0.6%, *Actinomadura* 0.4%), and (3) Dry Heating (*Streptomyces* 95%, *Nocardia* 2 %, *Kitasatospora* 2 % , *Micromonospora* 0.5 % , and *Amycolatopsis* 0.5 %). Pretreatment with 1.5% phenol reduced the number of non-*Actinomycetes* bacteria while significantly increased the number of *Micromonospora*-like colonies (Qiu *et al*. 2008). Phenol treatment of soil suspension lowered the number of fungi and other bacteria, but the *Actinomycetes* were less affected, thus 65% of the colonies belonged to rare *Actinomycetes*. The phenol

pretreatment of the soil killed bacteria and *Streptomycetes* in the samples, while keeping *Micromonosporae* and *Microbisporae* a live (Hayakawa *et al.* 1991). *Micromonospora* (49.18%), *Streptomyces* (24.59%), *Actinomadura* (13.11%), *Microbispora* (9.83 %), and *Polymorphospora* (3.27 %). This effect was also suggested in previous works (Hayakawa *et al*.1991; Hayakawa, 2008; Kim *et al*. 1994).

Phenols have been reported to exhibit antibacterial activities with distinguished characteristics in their reactivity with proteins related polyamides polymers (Haslam *et al*. 1996). The inhibition of microorganisms by phenolic compounds may be due to iron deprivation or hydrogen bonding with vital proteins such as microbial enzymes (Scalbert *et al*. 1991). The mechanism thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds possibly through reaction with sulfhydryl groups or though more nonspecific interaction with proteins. Phenolic compounds exert antimicrobial activity by injuring lipid-containing plasma membranes, causing leakage of cellular contents. The cell wall of rare *Actinomycetes* are rich in lipids, making them susceptible to the activity of phenol derivatives.

The dominance of other bacteria and fungal contamination inhibited the colonization of *Actinomycetes* on isolation medium. When antifungal agents such as nystatin (50 µg mL^{-1}) and cycloheximide (50 µg mL^{-1}) were supplemented into the isolation medium, the number of fungi decreased. Thus, the isolation medium was supplemented with these antibiotics in succeeding experiments (Seong *et al*. 2001).

Rare *Actinomycetes*' growth was disadvantaged when cultured on agar media together with other fast growing microorganism. Thus, the media for isolating them was designed to reduce growth of competitive microbes that it would not affect their growth. Hayakawa and Nonomura formulated Humic acid-Vitamin (HV) agar, a medium containing humic acid as the sole carbon and nitrogen source. Humic acid was extremely heterogeneous crosslinked polymers, which is resistant to biological decomposition. However, *Actinomycetes* can utilize it as nutrient source and also use it to supported sporulation, while it restricts the growth of non-filamentous bacteria colonies (Seong *et al.* 2001).

Humic Acid Vitamin Agar (HVA) was used for the selective isolation of *Actinomycetes*. The black color of HVA made it suitable to determine the morphology of white *Actinomycetes* colonies. Rare *Actinomycetes* as well as *Streptomyces* grew well on HVA. Although the growth rate of *Actinomycetes* is low, discrimination of typical morphology of colonies was easy on HVA. The activation of spore germination by humic acid was considered as one of the causes that increases the number of diverse *Actinomycetes* colonies on HV agar (Hayakawa and Nanomuraea 1987).

Phenol pretreatment as one of the improved selective isolation methods were applied successfully for isolating rare *Actinomycetes* from Indonesian soil. This method had been used for the selective isolation of genera *Micromonospora* (49.18%), *Streptomyces* (24.59%), *Actinomadura* (13.11%), *Microbispora* (9.83 %), and *Polymorphospora* (3.27 %). It was indicated that many novel *Actinomycetes* may be obtained by their exhibition of low similarity (< 98%) with their close relatives. Some of them may also be candidates for future potential novel bioactive compound producers.

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