Isolation of soil *Actinomycetes* from Forest Park of Pocut Merah Intan as potential producers of antimicrobial compounds

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Abstract. This study was undertaken to investigate soil *Actinomycetes* from grand forest park of Pocut Meurah Intan as well as to evaluate their potential to produce antimicrobial compounds. The study was conducted in Laboratory of Microbiology, Faculty of Mathematics and Natural Sciences, Syiah Kuala University from February to July 2012. The research was carried out by using an explorative method with laboratorial evaluation. Three different soil samples were taken from a depth of 10-20 cm below the soil surface and isolated on YM agar medium. During the investigation, 24 isolates were recovered and exhibited various morphological characteristics. The results of the antimicrobial assay showed that 14 isolates (58.33%) were active against one or more of the test organisms. Among the 14 active isolates, one isolate (7.14%) showed antibacterial activity; 9 isolates (64.29%) showed antifungal activity; and 4 isolates (28.57%) exhibited a broad-spectrum activity against both of the test bacteria and fungi. The inhibitory activity were also varied, ranged from weak (<5 mm), moderate (5-19 mm), to strong (>20 mm). The highest antimicrobial activity was presented by ATH-17 against *Candida albicans* (34.50 mm), while the lowest was exhibited by ATH-15 against *Escherichia coli* (0.20 mm).
Keywords: *Actinomycetes*, antimicrobial compounds, grand forest park.

Introduction

The ability of *Actinomycetes* to destroy microbial cells has been recognized since 1980 (Welsch, 1942), but the first antimicrobial compound derived from this bacteria was discovered in 1940th as Actinomycin (Waksman and Woodruff, 1941). Then, in 1944 Waksman discovered streptomycin (Miyadoh, 1990). Since then, the discovery of metabolite compounds from *Actinomycetes* increased greatly, reaching a peak in the 1970s, but declined rapidly in the late 1980s and 1990s (Watve *et al*., 2001). However, in response to the problems related to infectious diseases, such as the changing spectrum of pathogens (Cassell, 1997; Davies and Webb, 2004), drug resistance, patient's sensitivity and inability to control certain infectious diseases (Arasu *et al*., 2009), and immunodeficiency diseases (Madigan *et al.*, 2011), the screening of *Actinomycetes* for the production of novel antibiotics has been reincreased and in fact more intensively pursued by scientists.

In an effort to search for new secondary metabolites with antimicrobial activity, an exploration of previously unexplored habitats might be useful. A careful exploration of new habitats may lead to the discovery of different *Actinomycetes* taxa, and so may have the probability of discovering a new antimicrobial compounds (Hop *et al.*, 2011). One of the previously unexplored ecosystems for *Actinomycetes* is Grand Forest Park of Pocut Meurah Intan, Aceh, Indonesia. The forest has high geographical complexity ranging from lowland to mountainous land (0–400 m above sea level) and dominated by bladygrass (*Imperata cylindrica*), acacia (*Acasia auriculiformis*), and pinus (*Pinus mercusii*). Base on this background, the study was undertaken to isolate soil *Actinomycetes* from grand forest park of Pocut Merah Intan as well as to assess their antimicrobial activities.

Materials and Methods

Sample collection

Three diverse soil samples were collcted from grand forest park of Pocut Meurah, Saree, Aceh, Indonesia. Each sample represented soils of different vegetations, which were bladygrass (*Imperata cylindrica*), acacia (*Acasia auriculiformis*), and pinus (*Pinus mercusii*). Soil samples were taken from a depth of 10-20 cm below the soil surface. The soil of the top region (10 cm from the surface) was excluded (Aghighi *et al.*, 2004). The sample was transported aseptically in sterile petridishes to the laboratory and stored in the refrigerator at 4°C until use. The soil pH, temperature, and humidity were also determined.

Isolation and Purification of Actinomycetes

The soil samples were air-dried at room temperature for three days (Arasu *et al.*, 2009; Busti *et al.*, 2006). Samples of each soil were first mixed, suspended in sterile distilled water (10

g in 100 ml) and shaken on rotatory shaker (200 rev/min, 30 min). Portions (1 ml) of soil suspensions (diluted 10^{-1}) were transferred to 9 ml of sterile distilled water and subsequently diluted to 10^{-2} and 10^{-3}. Aliquots (0.1 ml) of 10^{-1}, 10^{-2}, and 10^{-3} were spread on the isolation plates containing Yeast Malt Extract Agar (ISP-2) (yeast extract 4 g, malt extract 10 g, glucose 4 g, and bacto agar 15 g, pH 7.2). To minimize the fungal growth, 2% of nystatin was added. The plates were incubated at 28 ^{o}C for seven to 14 days. Based on morphological features, the *Actinomycetes* cultures (rough, chalky) were selected and purified on new plate of ISP-2 medium at 28^{o}C for seven to 21 days.

Caracterisation of Actinomycetes Colonies

Morphological, physiological, and cultural features of the isolates were characterized according to methods of Shirling and Gottlieb (1966). Aerial mass color (mature sporulating aerial surface growth), substrate mycelia color (revese color), and diffusible soluble pigments on ISP-2 were determined after incubation at 27.0°C for 21 days using a reference color key according to Prauser (1964).

Preparation of Test Microorganisms

Antimicrobial activities were assayed against *Escherichia coli* ATCC 25922 (Gram-negative bacteria), *Staphylococcus aureus* ATCC 29213 (Gram-positive bacteria), and *Candida albicans* (yeast). Prior to the test, the bacteria were cultured on Nutrien Agar (NA) at 37^{o}C and yeast on Malt Extract Agar (MEA) at 30^{o}C for 24 h. The test bacteria and yeast were then subcultured in sterile nutrient broth (NB) and Yeast Glucose Chloramphenicol (YGC), respectively at room temperature in a rotary shaker (150 rpm) over night.

Antimicrobial Assay

The antimicrobial activity was determined by the plate diffusion method. One hundred mililiter (100 ml) of sterilized NA at 50^{o}C was seeded with 1 ml of each test bacteria and poured into petridishes, swirled gently and allowed to solidify. The same procedure was performed with 1 ml of yeast into 100 ml of sterilized MEA. From well-grown culture, agar discs of *Actinomycetes* colony mass were prepared using sterile cork borers. The discs were then aseptically transferred and equidistantly positioned on NA and MEA plates having fresh lawn cultures of bacteria and yeast, respectively. The dishes were pre-incubated at 4.0°C for 2 h to allow the diffusion of any antibiotics produced into the agar. After pre-incubation, the plates were incubated at 37°C for 24 h for *E. coli* and *S. aureus* and at 30°C for 48 h for *Candida albicans.* The antimicrobial activity was evaluated by measuring the inhibition zones diameter observed. Two replicates were carried out for each of the presumptive antagonist *Actinomycetes* isolates and mean value of inhibition zones was calculated.

Results and Discussion

Isolation of Actinomycetes

A total of 24 dferent *Actinomycetes* isolates were recovered from forest soil samples collected from preserved Grand Forest Park of Pocut Meurah Intan, Aceh, Indonesia, using Yeast Malt Extract Agar (YMA) supplemented with 2% of nystatin. All of the isolates were isolated at mesophilic temperatures (soil temperature 26^{o}C), acidophilic condition (soil pH 5.6), and relatively high soil moisture (relative humidity 50%).

The number of *Actinomycetes* isolates recovered from soil samples varied widely, from less than 5 isolates (Kavitha *et al.*, 2010) to more than 1000 isolates (Hayakawa *et al.*, 2010). Their presence in soils is greatly influenced by many factors such as the enviromental conditions of pH, temperature, humidity, and vegetation (Basilio *et al.*, 2003), pretreatment of soil samples before isolation (Hayakawa *et al.*, 2010) and cultural condition (Basilio *et al.*, 2003). In addition, type of soils and soil components also affect the occurance of *Actinomycetes* in soils (Barakete *et al.*, 2002; Thakur *et al.,* 2007).

Morphological and cultural characteristics of Actinomycetes isolates

All isolates that grew on YMA showed morphology typical of *Actinomycetes* since the colonies were slow growing, glabrous or chalky, folded, and with aerial and substrate mycelia of different colors (Locci *et al.*, 1989). In addition, all colonies possessed an earthy odour. Some of them also produced antimicrobial compounds as reflected by zones of growth inhibition among other inhabitants of soil samples. The cultural characteristics (pigment production) and morphological characteristics of each *Actinomycetes* isolates are presented in Table 1.

Table 1. Morphological and cultural characteristics of the *Actinomycetes* isolates on ISP-2

Isolates code	Color of Aerial Mycelium	Color of Substrate Mycelium	Soluble Pigment
ATH-01	Grey	Brown	Brownish yellow
ATH-02	Variable (grey, white)	Dark Brown	
ATH-03	Variable (grey, white)	Cream	
ATH-04	White	Brown	Brownish red
ATH-05	Green	Brown	Brownish red
ATH-06	Dark brown	Black	
ATH-07	White	Cream	
ATH-08	White	Cream	
ATH-09	White	Brick-Red	
ATH-10	White	Brick-Red	Brick-red
ATH-11	Light grey	Dark Brown	Brownish green
ATH-12	White	Cream	
ATH-13	White	Brick-Red	Brownish yellow
ATH-14	Variable (cream, white)	Cream	
ATH-15	White	Cream	
ATH-16	Orange	Orange	
ATH-17	Cream	Cream	
ATH-18	Cream	Cream	
ATH-19	Yellowish cream	Dark Brown	Dark brown
ATH-20	White	Cream	
ATH-21	White	Black	Pinkish red
ATH-22	White	Cream	Brick-red
ATH-23	Light brown	Cream	
ATH-24	No aerial mycelia	Cream	

All of these isolates fitted the *Actinomycetes* description as reported by several investigators (Shirling and Gottlieb, 1966). The colour of the aerial spore mass and substrate mycelium was varied. The aerial mycelium colours, determined based on the colour of their mature sporulated aerial mycelium, were categorized into eleven colour series with white colour being the most dominant (45.83%), followed by grey and white colour combination (8.33%), cream (8.33%), grey (4.17%), green (4.17%), orange (4.17%), light brown (4.17%), dark drown (4.17%), variable of cream and white (4.17%), and yellowish cream (4.17%), while one isolate did not produce aerial mycelia. The highest occurrence of isolates of the white series is in agreement with that reported by many authors (Thakur *et al*., 2007; Masmeh, 1992). Nevertheless, Barakete *et al*. (2002) in his study on characterization of rhizospheric soil S*treptomycetes* from Moroccan habitats, reported that the grey colour class dominated (40%) of 131 isolates.

To examine the reverse side colour and soluble pigment on the media, the direction recommended by Shirling and Gottlieb (1966) was used. Out of 24 *Actinomycetes* isolates, 20 (80%) isolates showed distinctive reverse side pigment, and 9 (24%) isolates produced soluble pigments (Table 1). The variety in morphological, cultural, and physiological characteristics of the obtained *Actinomycetes* indicated that the isolates investigated from Grand Forest Park of Pucut Meurah Intan consist of more than one species. Barakete *et al.* (2002) sugested that the differences in colour of the aerial mycelia of the isolates as well as those of the pigments they produce, may be an indication of the diversity of *Actinomycetes* isolates in certain sites of investigation.

Antimicrobial Activities

The total number of isolated *Actinomycetes* (24 isolates) was screened on agar medium and the antimicrobial activity presented by the isolates was varied. Fourteen (58.33%) of twenty four *Actinomycetes* isolates were shown to have very potent antimicrobial activity against at least one of the target microorganisms. The results of the antimicrobial activity of the active isolates are shown in Figure 1.

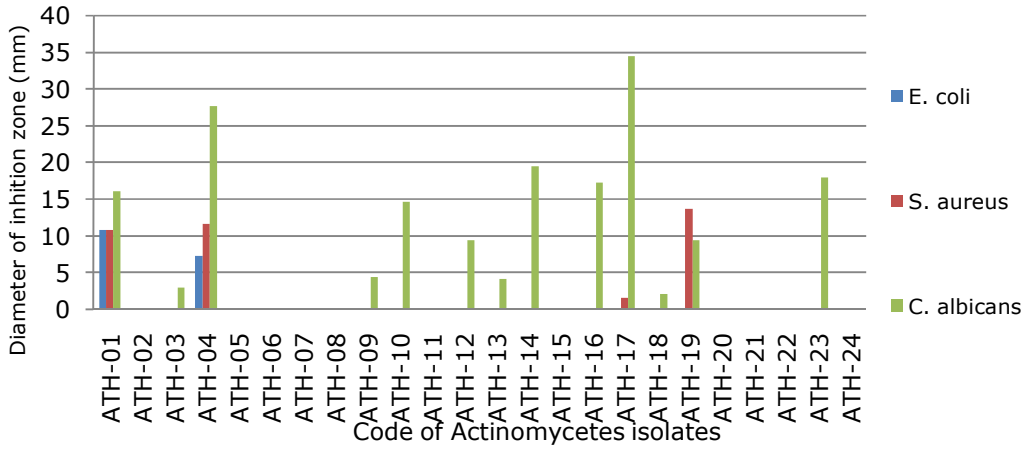

Figure 1. Antimicrobial activity of *Actinomycetes* isolates

Among the active isolates displayed in Figure 1, antibacterial activity was observed only in one isolate (7.14%), 9 isolates (64.29%) presented antifungal activity, and 4 isolates (28.57%) exhibited a broad-spectrum of antibacterial and antifungal activity. It is clear that the best percentage (60.86%) of antimicrobial activity found in the present study was antifungal activity. This percentage is in contradiction with the observation of many authors studying the antimicrobial activity of soil *Actinomycetes*, who reported that antibacterial activities of *Actinomycetes* isolates were higher than those of antifungal (Salamoni *et al.*, 2010; Kitouni *et al.*, 2005; Basilio *et al.*, 2003).

These differences in percentage of antibiosis may imply that the investigated *Actinomycetes* isolates belong to different species or to the same one but they produced different bioactive compounds, especially that with antifungal activity. According to Nonoh *et al.* (2010) and Thakur *et al.* (2007), *Actinomycetes* isolates obtained from soil samples collected from preserved forest ecosystem showed a broad-spectrum of antifungal activities. In addition, a preserved forest, as protected from human activity, is an exellent condition for *Actinomycetes* growth, so that it enhances the competition for survival and the production of powerful vital substances. Other assumption of this difference in antagonistic activities is that due to the difference in medium used for the antimicrobial assays. Arasu *et al.* (2008) suggested that production of antimicrobial compounds is strongly affected by the nature of medium composition.

Furthermore, results of antibiotic activity expressed in terms of the diameter of the inhibition zone were varied and ranged from weak to strong. The strongest antagonistic activity was noticed in isolate ATH-17 against *C. albicans* (34.50 mm) and the weakest one was exhibited by isolate ATH-15 against *E. coli* (0.20 mm), which was also noticed as the only isolate that showed only antibacterial activity (Figure 1). On the basis of the target microorganism, the strongest sensitivity toward the active *Actinomycetes* isolate was also showed by *C. albicans* with mean of inhibition zones 13.89 mm, followed by *S. aureus* (9.48 mm), and the lowest sensitivity was exhibited by *E. coli* (6.17 mm). Acording to Rante *et al*. (2010) antimicrobial activity is caegorized as weak (<5 mm of inhibition zone); moderate (5-19 mm), and strong (>20 mm).

The nine (9) isolates (Figure 1) which were only active against *C. albicans* were identified with the following codes: ATH-03, ATH-09, ATH-10, ATH-12, ATH-13, ATH-14, ATH-16, ATH-18, and ATH-23 and the inhibition zones observed were 3.00 mm, 4.40 mm, 14.65 mm, 9.48 mm, 4.18 mm, 19.5 mm, 17.35 mm, dan 2.15 mm, and 18.00, respectively. Base on these diameter of inhibition zones produced, their ability to inhibit growth of fungi showed weak (<5 mm inhibition zone) and moderate (5 – 19 mm) antibiosis.

Broad spectral activity was noticed in isolates ATH-01, ATH-04, ATH-17, and ATH-19. Isolates ATH-17 and ATH-19 showed broad-spectrum activity against *S. aureus* and *C. albicans*, while isolates ATH-01 and ATH-04 presented a broad-sprectum against all of the three test microorganisms (*E. coli*, S*. aureus*, and *C. albicans*). The extent of antibiosis of these broad-spectral *Actinomycetes* isolates against the test organisms is different; Isolate ATH-01 exhibited moderate antibiosis against all of the test organisms (10.8 mm, 10.85 mm, and 16.15 mm

against *E. coli*, S. aureus, and *C. albicans*, respectivey), while ATH-04 showed moderate activity against *E. coli* (7.35 mm) and S. aureus (11.70 mm), but strong activity against *C. albicans* (27.68 mm). On the other hand, weak antibiosis was noticed in ATH-17 against *E.coli* (1.65 mm), but was strong against *C. albicans* (34.50 mm). Finaly, moderate activity was presented by ATH-19 against both *E. coli* (13.7 mm) and *S. aureus* (9.45 mm).

Among the *Actinomyetes* that showed activities on bacteria, high percentage of inhibition was recorded against Gram-positive bacteria, while Gram-negative test bacteria were less inhibited. Many authors also reported that *Actinomycetes* isolates appear to be highly active against gram-positive bacteria (Oskay *et al.*, 2004; Ambarwati and Gama, 2009; Barakete *et al.*, 2002; Basilio *et al.* 2003). The reason for the difference in sensitivity between Gram positive and Gram negative bacteria could be attributed to the morphological differences between these two group microorganisms, Gram negative bacteria have an outer polysaccharide membrane carrying the structural lipopolysaccharide components, which makes the cell wall impermeable to lipophilic solutes, unlike the Gram positive bacteria that has only an outer peptidoglycan layer which is not an effective permeability barrier (Cwala *et al.*, 2008). Ten (41.67%) isolates of the the total 24 *Actinomycetes* isolates obtained did not show any antimicrobial activity towards the test organisms, but it is probable that they produce other useful compounds for which they were not screened in this study. In fact, Porter (1971) stated that probably all *Streptomycetes* possessed some antimicrobial properties if proper conditions were taken into consideration during culturing of these organisms for purposes of assessing their antibiotic production.

Conclusions

A total 24 isolates were recovered from the soil samples collected from Grand Forest Park of Pucut Meurah Intan and exhibited different morphological, physiological, and cultural characteristics. Of the 24 isolates, 14 isolates (58.33%) showed antimicrobial activities against at least one of the test microorganisms; one isolate showed antibacterial activity; nine isolates showed antifungal activity; and four isolates exhibited a broad-spectrum activity against both of the test bacteria and fungi. The inhibitory activity also varied, ranged from weak (<5 mm), moderate (5-19 mm), to strong (>20 mm). Nevertheles, most of the active isolates showed moderate activity. The highest antimicrobial activity was showed by ATH-17 against *Candida albicans* (34.50 mm), while the lowest was exhibited by ATH-15 against *Escherichia coli* (0.20 mm).

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