

## Diversity of antibiotic-producing Actinomycetes in mangrove forest of Torosiaje, Gorontalo, Indonesia

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**Abstract.** Retnowati Y, Sembiring L, Moeljopawiro S, Djohan TS, Soetarto ES. 2017. Diversity of antibiotic-producing actinomycetes in mangrove forest of Torosiaje, Gorontalo, Indonesia. *Biodiversitas* 18: 1453-1461. Actinomycetes for antibiotic production have been studied at various extreme environments. Mangrove forest of Torosiaje in Gorontalo Province, Indonesia has unique geomorphological conditions where the forest is surrounded by karst ecosystem consisting of fringe and overwash types. Therefore, the objective of this study was to analyze the distribution and diversity of antibiotic-producing Actinomycetes in various rhizosphere including different locations and mangrove species. Samples of rhizosphere soil were collected in the depth of 0-10 cm, which was then subjected to detailed physicochemical analysis. Actinomycetes were collected through heating pre-treatment (60°C for 15 min) followed by culturing in the Starch Casein Agar medium supplemented with cycloheximide and nystatin. The screening process of antibiotic-producing Actinomycetes was based on Agar block method against pathogenic microorganisms. Grouping of Actinomycetes was determined by ARDRA fingerprinting analysis. The diversity of Actinomycetes was analyzed based on sequencing of 16S rDNA. The results showed that the distribution of Actinomycetes was found in overwash type, middle zone and upper zone of fringe type including rhizosphere of 7 species of mangrove. The highest population of Actinomycetes was found in rhizosphere of *R. mucronata* at the overwash type, and the lowest one found in rhizosphere of *R. apiculata* at the middle zone of fringe type. A total of 77 isolates amongst 167 isolate collection showed antibacterial activities. Forty seven representatives from 77 antibacterial-activities isolates were selected using ARDRA for partial characterization according to their phylogenetic diversity. Sequencing and analysis of 16S rDNA from selected-representative isolates displayed the presence of members associated with Actinomycetes genera such as *Streptomyces*, *Amycolatopsis*, *Saccharomonospora*, and *Nocardiopsis*. The member of genus *Streptomyces* such as *Streptomyces qinglanensis* and *Streptomyces champavatii* were distributed across locations. Genus *Saccharomonospora* and *Nocardiopsis* were mostly found at the overwash type, while *Amycolatopsis* was found at the upper zone of fringe type.

**Keywords:** Actinomycetes, diversity, fringe, mangroves, overwash

### INTRODUCTION

Actinomycetes are Gram-positive bacteria that produce bioactive compound (Amrita et al. 2012). 70-80% of bioactive compounds are produced by Actinomycetes (Kanna et al. 2011), especially *Streptomyces* (Berdy 2005; Chaudary et al. 2013), and a part of bioactive compounds is produced by rare-Actinomycetes (Berdy 2005). The exploration of Actinomycetes that produce bioactive compounds has been focused on the extreme environment due to the rediscovery of bioactive compounds from terrestrial environment (Nolan and Cross 1988; Mangamuri et al. 2014). Mangrove forest is one of interesting ecosystems to explore Actinomycetes (Santhi et al. 2010; Baskaran et al. 2011; Khanna et al. 2011; Naikpatil and Rathod 2011; Ravikumar et al. 2011; Mangamuri et al. 2012; Mangamuri et al. 2014).

Mangrove ecosystem is a habitat of various of Actinomycetes. The Actinomycetes of mangrove ecosystem produce 122 bioactive compounds, where 73 of them have been identified as new kinds of bioactive

compounds, and 49 compounds have not been identified, yet (Xu et al. 2014). The bioactive compounds from mangrove Actinomycetes demonstrate antibacterial activity (Sharma et al. 2011; Hunadanamra et al. 2013; Das et al. 2014; Phongsopitanum et al. 2014), anti fungal activity (Suthindiran and Kannabiran 2010; Sharma et al. 2011; Phongsopitanum et al. 2014; Das et al. 2014), and anti viral activity (Waksman 1984) and anti cancer activity (Hong et al. 2009; Suthindiran and Kannabiran 2010; Becerril-Espinosa et al. 2012; Ravikumar et al. 2012; Anupa et al. 2013; Xu et al. 2014).

Actinomycetes producing bioactive compounds in mangrove ecosystem are distributed on rhizosphere and non rhizosphere (Xiao 2009; Ravikumar et al. 2011), which rarely live as endophytes on stems and leaves of mangrove (Ravikumar et al. 2011; Gayathri and Muralikrishnan 2013; Govindasamy and Franco 2013). The number of Actinomycetes population in rhizosphere are more than those in non rhizosphere. The various number of Actinomycetes is also depended on types of mangrove (Balagurunathan et al. 2010; Ravikumar et al. 2011;

Ramirez-Elias et al. 2014).

Mangrove ecosystem of Torosiaje is karst mangrove ecosystem including two types of forest, i.e., fringe and overwash types. The fringe type is divided into two zones, i.e. middle and upper zone. The middle zone was dominated by *Rhizophora apiculata* and *Bruguiera gymnorrhiza* while the upper zone consists of *Sonneratia alba*, *Avicennia marina*, *Xylocarpus* sp., and *Ceriops tagal*. The overwash type consists of *Rhizophora mucronata* and *Bruguiera gymnorrhiza* (Katili et al. 2014). Based on the uniqueness of Torosiaje mangrove ecosystem and some other important aspects; however, the diversity and distribution of Actinomycetes in this location had not been comprehensively explored, yet. Therefore, the objective of this study was to analyze the diversity and distribution of antibiotic-producing Actinomycetes in rhizosphere of various species mangrove located at mangrove ecosystem of Torosiaje, Gorontalo, Indonesia.

## MATERIALS AND METHODS

**Study area and sampling of soil rhizospheres.** This study was conducted from April 2016 to April 2017. Samples of mangrove rhizospheres soil were collected from mangrove forest at Torosiaje, Pohuwato District, Gorontalo Province, Indonesia (Figure 1). The total of eight soil samples was collected from 3 different location i.e overwash type, middle zone and upper zone of fringe type, including 7 species of mangrove rhizospheres (*Rhizophora mucronata*, *Rhizophora apiculata*, *Bruguiera gymnorrhiza*, *Avicennia marina*, *Xylocarpus* sp., *Ceriops tagal* and *Sonneratia alba*). Sampling sites were located at 3 different coordinates i.e N. 00°28'21,9''; E.121°26'55,7''; N. 00°28'50,5''; E. 121°27'56,8'' and N. 00°28'47,2; E. 121°27'56,2''. Soil samples were collected on the depth of 0-10 cm by modified soil core with 4 replication.

**Soil physicochemical characteristic analysis.** The physicochemical analysis of soil samples (salinity, soil texture, soil pH, C-organic, total-N, N-NO<sub>3</sub>, Fe, Mn, Zn, Cu, phosphor, K<sub>2</sub>O and total S) was conducted at Soil Chemical laboratory, Faculty of Agriculture, Gadjah Mada University, Yogyakarta, Indonesia.

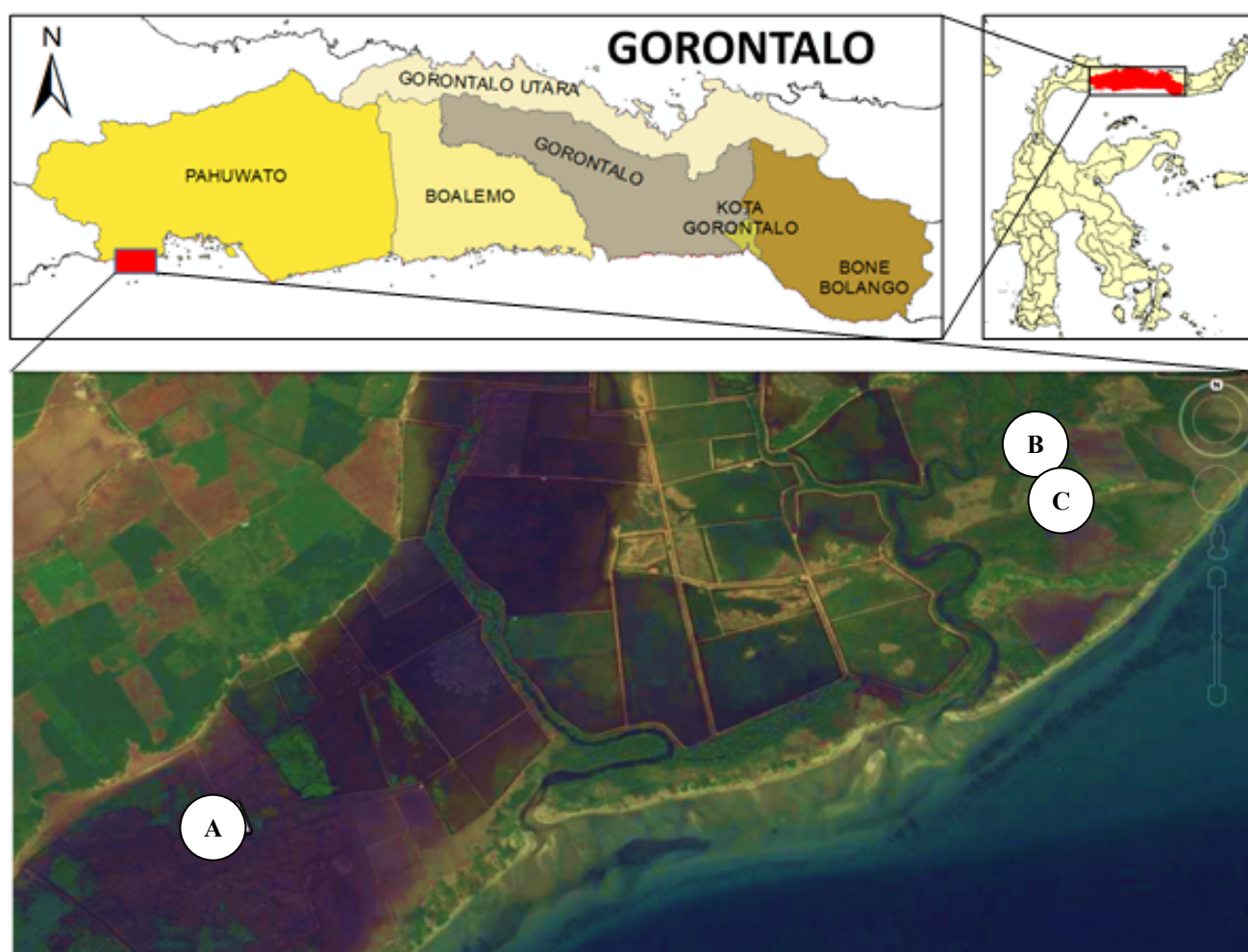
**The isolation of actinomycete isolates.** The initial treatment of rhizosphere soil samples was based on dry wet method on 60°C for 15 minutes in combination of sea water and aquadest (1: 1 v/v) (Mangamuri et al. 2012). Soil suspension was serial-diluted up to 10<sup>-6</sup>. Soil suspension as much as 100 µL was inoculated in Starch Casein Agar (SCA) medium supplemented by 25 µg/mL of cycloheximide and 25 µg/mL of nystatin (Baskaran et al. 2011). Then incubated on 28 ± 2 °C for 10-28 days. Colony Forming Unit (CFU) was calculated and isolated in slant ISP 2 medium.

**Screening of actinomycete isolates for potential antibiotics production.** The screening of Actinomycetes for potential antibiotics production was based on Agar Block Method. The medium Muller Hinton agar got 5 holes and each hole was 5 mm diameters. After 28 days of age, actinomycete isolates cultured on ISP2 medium were then

clipped around 5 mm diameters and moved to the holes. The Actinomycetes were incubated on 28°C ±2 for 3-7 days. The cell mass of microorganisms test (*Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*) on Nutrient Broth medium was equated by spectrophotometry on OD 0,6. Afterward, 300 µL of microorganisms test was inoculated in the around of actinomycete growing by surface plate method, then incubated on 37°C for 2 x 24 hours.

**Extraction of genomic DNA.** The spores of actinomycete isolates were suspended in aquadest. The suspension of spore was inoculated in 20 mL of ISP2 broth, then incubated in shaker incubator at 150 rpm for 4 to 7 days. Cell pellet in 20 mL of ISP2 broth was separated by centrifugation at 13.000 rpm for 5 min. The cell pellet was added with 20 mg of glass bead and 1 mL of buffer lysis (100mM Tris HCl pH 8, 50 mM EDTA pH 8, 2% SDS and 100 mM NaCl) then shaken out vigorously by vortex for 2 min. Proteinase K (20 mg/mL) as much as 10 µL was added then shaken out vigorously for 30 min. Then 60 µL of lysozyme (100 mg/mL) was added and incubated on 55°C for 30 min in water bath. The suspension was centrifugated at 13.000 rpm for 5 min, then cell pellet was discarded and the supernatant was resuspended by phenol (1: 1 v/v). The suspension of supernatant and phenol were shaken vigorously for 30 minutes, then centrifugated on 13.000 rpm for 10 min. The top layer was moved to new microtube and added by cold chloroform (1: 1 v/v), then shaken out slowly for 20 minutes. Then they were centrifugated on 13000 rpm for 10 minutes. The top layer was moved to new microtube and added with cold absolute ethanol (1: 1 v/v), and saved on -80°C for 2 hours or -20°C for overnight. The DNA genome pellet was separated by centrifugation at 13000 rpm for 10 minutes, cleaned up with 70% cold ethanol then dried for 2 hours. The DNA genome was suspended in 50 µL of TE (pH 8). The suspension of DNA genome was added by 3 µL of RNAase (10µg/mL) and incubated on 37°C for 1 hour in water bath. The genomic DNA was applied or saved on -20°C. The genomic DNA were examined by 2% agarose gel electrophoresis. The gels were stained by syber and visualized under UV transilluminator (Magarvey et al. 2004).

**Amplification of 16S rDNA.** The 16S rDNA was amplified from genomic DNA samples using eubacterial universal primers 27 F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3'). The amplification was carried out in 25 µL volume by using 1 µL of genomic DNA as template with 12.5 µL GoTaq (R) Green Master Mix [buffer reaction, deoxynucleotide triphosphate (dNTPs), Taq DNA polymerase, and MgCl<sub>2</sub>], 9.5 µL dH<sub>2</sub>O, and 1 µL universal primer 27F and 1 µL 1492R. The thermal cycling conditions were as follows: initial denaturation at 96°C for 5 min; 29 cycles at 95°C for 1 min, 54°C for 1 min, 72°C for 2 min and post-extension at 72°C for 10 min. The amplification reaction was performed by Bio-Rad thermal cycler (MyCycler, Bio-Rad, USA) and the amplified products were examined by 2% agarose gel electrophoresis (Mangamuri et al. 2012).



**Figure 1.** Location of mangrove forest of Torosiaje, Gorontalo, Indonesia. The alphabet A, B and C indicating the sampling site of mangroves soil rhizosphere at different mangrove forest type. A. overwash type (N. 00°28'21,9"; E.121°26'55,7"), B. middle zone of fringe type (N. 00°28'50,5"; E. 121°27'56,8"), and C. upper zone of fringe type (N. 00°28'47,2; E. 121°27'56,2")

**Amplified ribosomal DNA restriction analysis (ARDRA).** To identify the number of polymorphic groups and to select representative strains among the actinomycete isolates, aliquots of purified 16S rDNA amplicons were subjected to amplified ribosomal DNA restriction analysis. The purified PCR products were digested separately with *Hae*III and *Msp*I, in 10 µL reaction volume by using manufacture's recommended buffer and temperature. The digested restriction fragments were electrophoresed in 2% agarose gels using TE buffer. The gels were stained with syber and visualized under UV transilluminator. Strong and clear bands were scored in a binary data used for similarity and clustering analysis in numerical taxonomy analysis program package, MVS Plus (Multivariate Statistical Package) Version 3.1A. Similarities among the isolates were calculated by Jaccard's coefficient, and the dendrogram was constructed using UPGMA (Unweighted Pair Group Method with Arithmetic Averages) method (Jose and Jebakumar 2012). If the strains formed a cluster

defined at the 72% similarity level, they would be assigned to a single species (Meng Yuan et al. 2014)

**16S rDNA sequencing and phylogenetic analysis.** The PCR amplicons of 47 actinomycete isolates with representative ARDRA profiles were purified by using PCR product purification spin kit (BigDye® Terminator v3.1, USA) and sequenced by ABI PRISM 3730xl Genetic Analyzer develop by Applied Biosystems using same primary set as used in PCR amplification. All the sequences obtained from sequencing phase were analyzed and edited by using BioEdit soft-ware (Jose and Jebakumar 2012). Initially, all the 16S rDNA sequences were compared to sequences in GenBank by using the online service of Basic Local Alignment Search Tool to determine the approximate phylogenetic position. Sequences were aligned by using ClustalW with representative actinomycete 16S rDNA sequences, and a phylogenetic tree was constructed by using the Molecular Evolutionary Genetics Analysis (MEGA) software, version 6.06 (Yuan et al. 2014).

## RESULTS AND DISCUSSION

### Physico-chemical characteristics of soil, population and distribution of Actinomycetes

Mangrove forest ecosystem is a habitat for various types of tolerant microorganisms to an extreme environmental conditions. Mangrove forest ecosystems are rich in organic, nitrogen and sulfur materials that required for the grow of microorganisms (Malek et al. 2014). Mangrove ecosystem of Torosiaje, Gorontalo consist of two type mangrove forest i.e., overwash type and fringe type. Fringe type divided into 2 zone i.e., middle zone and upper zone. Each of mangrove forest type and zone were show the specific physicochemical characters. The sediment salinity level was in range of 23,1 - 31,3 ppt. The physico-chemical characteristics of soil samples collected from overwash mangrove, middle and upper zone of fringe mangrove at mangrove forest of Torosiaje, Gorontalo were studied using standard protocols and summarized in Table 1. Several parameters, such as pH, salinity of soil, soil texture, macroelements and microelements have been measured. The results show that there were differences across all measurable characters in all three locations. Overwash type mangroves forest is washed periodically by tides of sea water so that it affects to the physicochemical character of the soil to be quite extreme, including salinity and high level of Fe, while the organic-C content was relatively low. Middle zone and upper zone in fringe type mangrove forests were shown the differences physico-chemical characters amongst both of them. The upper zone tends to be at least exposed by sea tides was shown sandy soil conditions with low salinity and neutral pH.

The physicochemical character of the 3 sampling sites were responded by actinomycete as one of the members of the mangrove ecosystem. The responses of actinomycete were indicated by the various number of population in the three locations. The highest population of Actinomycetes was found on overwash type, and the lowest one was found on middle zone of fringe type (Table 2). The periodically tides of overwash type is causes a continuously flow of macro-element and micro-element that supporting the growth of soil microorganisms. Otherwise, the geographical condition of the sloping fringe mangrove forest causes the middle zone is more exposed by the tides than the upper zone. This condition might cause the nutrient in the middle zone was more transported out of the zone.

Actinomycetes exploration at mangrove forest of Torosiaje is more emphasized on rhizosphere soil samples of 7 types mangroves at the depth of 0-10 cm. Nevin et al. (2000) and Mangamuri et al. (2011) claimed that the most Actinomycetes population were found in mangrove rhizosphere at the depth of 0-20 cm and apparently varied in each species of mangroves. The high population of Actinomycetes in the rhizosphere was due to the root exudation activities. That activity provided the space for actinomycete colonization and depended on the availability of amino acids and high concentrations of sugars and low concentrations of tannin content in rhizosphere (Atlas and Barta 1998; Badri and Vivanco 2009; Mangamuri et al. 2011). Mangamuri et al. (2011) also reported that the

**Table 1.** Physico-chemical characteristic of soil samples from overwash type, middle zone and upper zone of fringe type at mangrove forest of Torosiaje, Gorontalo, Indonesia

Physicochemical characters of soil	Fringe type		Overwash type
	middle zone	Upper zone	
Salinity (ppt)	26.56	23.17	31.3
pH	3.16	7.46	6.04
Soil texture:			
Sand (%)	62	84	62
Dust (%)	31	10	26
Clay (%)	7	6	12
C-organic (%)	12.63	23.33	2.18
N (ppm)	102.94	246.65	610.40
Total-S (%)	0.38	0.20	0.43
N-NO <sub>3</sub> (ppm)	24.09	66.44	62.31
P <sub>2</sub> O <sub>5</sub> (ppm)	63.33	21.31	3.09
K (me/100g)	1.74	3.59	1.23
Fe (ppm)	427.69	120.09	441.24
Mn (ppm)	4.76	4.04	8.08
Zn (ppm)	4.31	0.70	11.03
Cu (ppm)	0.37	0.76	1.07

**Table 2.** Population of Actinomycetes at 7 types mangrove rhizosphere

Mangrove forest type	Zone	Mangrove type	Actinomycete population (CFU/g)
Overwash	-	<i>R. mucronata</i>	23,5 x 10 <sup>6</sup>
		<i>B. gymnorhiza</i>	78,4x10 <sup>3</sup>
		Total on overwash	11,8 x 10 <sup>6</sup>
Fringe	Middle	<i>R. apiculata</i>	3,5 x 10 <sup>1</sup>
		<i>B. gymnorhiza</i>	10,1 x 10 <sup>3</sup>
	Sub total		50,7 x 10 <sup>2</sup>
	Upper	<i>S. alba</i>	34 x 10 <sup>1</sup>
		<i>A. marina</i>	33,6 x 10 <sup>3</sup>
		<i>Xylocarpus</i>	26,3 x 10 <sup>2</sup>
		<i>C. tagal</i>	18,6 x 10 <sup>2</sup>
	Sub total		96,1 x 10 <sup>2</sup>
		Total on fringe	73,4 x 10 <sup>2</sup>

important nutrients such as nitrogen and potassium were found to be highest in the soil depths of 0-10 cm.

The isolation of actinomycete from mangrove rhizosphere using Starch Casein Agar medium supplemented with cycloheximide and nystatin was succeeded obtaining 167 actinomycete isolates. The type of mangrove vegetation affected the distribution and population of actinomycete (Balagurunathan et al. 2010; Ramirez-Elias et al. 2014; Ravikumar et al. 2011; Ravikumar et al. 2012). The result of study show that the number of actinomycete isolate was varied in the 7 types of mangrove rhizosphere (Figure 2). The highest number of isolate was found at *R. mucronata* rhizosphere and the lowest one at *R. apiculata* rhizosphere. This is probably due to the rhizospheres effect as a result of root exudation activity. The root exudates contains various compounds including amino acids, organic acids, sugars, phenols,

tannins, alkaloids and various secondary metabolites (Atlas and Barta 1998; Walker et al. 2003). Exudation activities of various compounds in plant roots can regulate the soil microbial community by mediating soil-microbial root interactions, altering soil chemical physical properties and inhibiting the growth of plant competitors (Badri and Vivanco 2009). Each of mangrove species was produce a specific kind of root exudates. The adult of *Avicennia marina* is produce root exudates with different levels of glucose and fructose composition (Lie et al. 2014) and root exudates of *Bruguiera gymnorhiza* contain of aminopyrine, palmitic acid, stearic acid, di-n-propyl-ether, and 2.5-angidrolunonic (Kumar et al. 2009).

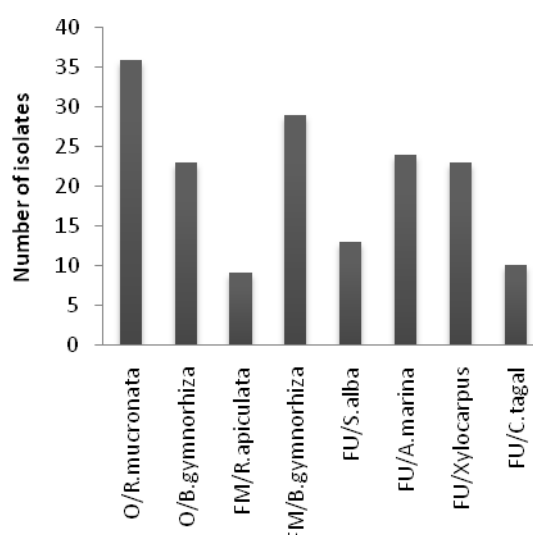
### Screening of antibiotic-producing Actinomycetes based on agar block method

Mangrove forests are interesting ecosystems for the discovery of antibiotic-producing Actinomycetes (Santhi et al. 2010; Baskaran et al. 2011; Khanna et al. 2011; Naikpatil and Rathod 2011; Ravikumar et al. 2011; Mangamuri et al. 2012; Mangamuri et al. 2014). The extreme conditions of mangrove environment are expected to stimulate the actinomycete to produce antibiotics with specific character (Basilio et al. 2003). The bioactive compound were shown the activity as antibacterial, antifungal, antiviral and anticancer (Suthindhiran et al. 2010; Ravikumar et al. 2011; Amrita et al. 2012; Doroghazi and Metcalf 2013; Zotchev 2014). This study proves that amongst 167 actinomycete isolates, there were 77 actinomycete isolates that show the antibacterial activity against pathogenic bacteria. The highest number of antibiotic-producing actinomycete isolates was found at rhizosphere of *R. mucronata* in overwash type, and the

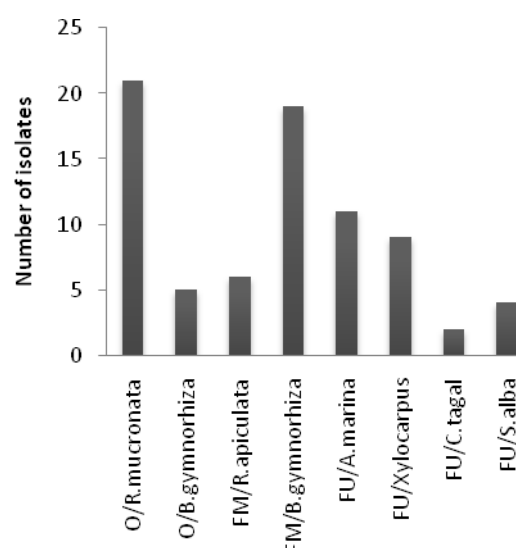
lowest one at rhizosphere of *C. tagal* in upper zone of fringe type (Figure 3). While the high ability of actinomycete isolates to against pathogenic microbes was found in rhizosphere of *A. marina* and *Xylocarpus* sp. at upper zone of fringe type. That was shown that mangrove forest of Torosiaje, Gorontalo was potential to exploration antibiotic-producing Actinomycetes.

**Amplification of 16S rDNA and ARDRA.** PCR amplification of the 16S rDNA using a set of universal eubacterial specific primers: 27 F and 1492R yielded a single amplicon of ~1500 bp for 77 the antibiotic-producing actinomycete isolates. The amplicons of 16S rDNA were used to Amplified Ribosomal DNA Restriction Analysis (ARDRA) with restriction enzymes endonuclease *HaeIII* and *MspI*. Digestion of these amplicons with *HaeIII* were generated almost similar profile for several of isolates. Whereas, when amplicons were digested by restriction enzyme *MspI*, different number of fragments were produced about 5 to 9 fragments with varied sizes about 30 to 900 bp for different isolates.

The ARDRA pattern was used for dendrogram construction using UPGMA and Jaccard's coefficient algorithms (Figure 4). The 77 of antibiotic-producing actinomycete isolates were divided into 47 groups in a dendrogram inferred from ARDRA pattern obtained from *MspI* restriction enzymes. The groups consisted of 1 to 5 actinomycete isolates. The groups formed on the dendrogram provide information about the presence of the same species found in different location and different kinds of mangrove. If the isolates formed a group/cluster defined at the 72% similarity level, they would be assigned to a single species (Meng Yuan et al. 2014).



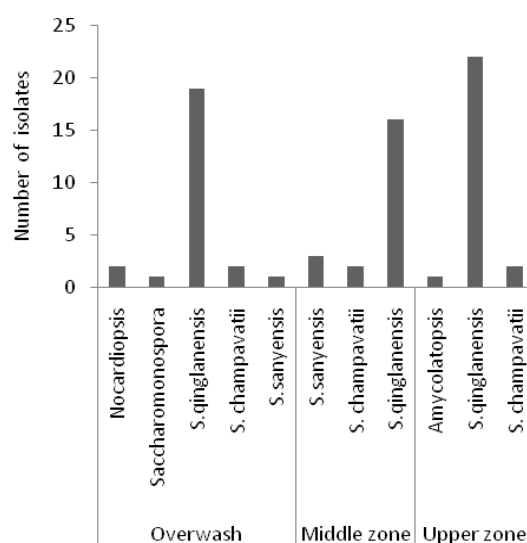
**Figure 2.** The number of Actinomycete isolates in 7 types of mangrove rhizosphere at mangrove forest of Torosiaje Gorontalo, Indonesia. O. Overwash; FM. Fringe/middle zone; FU. Fringe/upper zone



**Figure 3.** The number of antibiotic-producing Actinomycete isolates at 7 kind of mangrove rhizosphere. O. Overwash; FM. Fringe/middle zone; FU. Fringe/upper zone

**Actinomycetes community composition and phylogenetic analysis.** The 16S rDNA of 47 representative isolates that belong to different clusters established by ARDRA was sequenced and used to determine the diversity of isolates. Based on similar criteria of 98% at the 16S rRNA gene, the 47 isolates were sorted into 6 phylotypes, belong to 4 different genera. The four genera were *Streptomyces*, *Saccharomonospora*, *Nocardiopsis* and *Amycolatopsis* (Figure 5). The most abundant group of isolates was affiliated with the genus *Streptomyces*, represented by four ribotypes accounting for 95% of the total actinomycete population. The ribotypes ORm2-a was closely related to type strain *Streptomyces qinglanensis* (GenBank: NR\_109303.1) with 99.9% sequence similarity, representing 83% of total isolates. The other ribotypes of *Streptomyces*, OBg1-3 (representing 5% of isolates) and FMRa1-1 (representing 7% of all isolates) were closely related to type strain *Streptomyces sanyensis* (GenBank: NR\_116599.1) with 99% sequence similarity and *Streptomyces champavatii* (GenBank: NR\_115669.1) with 97% sequence similarity, respectively. The second most dominant group in the isolates was the genera *Nocardiopsis*, representing 3% of total actinomycete isolates. Ribotype ORm2-f was closely related to type strain *Nocardiopsis lucentensis* (GenBank: NR\_026342.1) with 99% of sequence similarity. Ribotype FUXy2-d and OBg4-e representing 1 % of total actinomycete isolates, respectively. Ribotype FUXy2-d was closely related to type strain *Amycolatopsis sacchari* (GenBank: NR\_112696.1) with 94% of sequence similarity. While, ribotype OBg4-e was closely related to type strain *Saccharomonospora amisosensis* (GenBank: NR\_109529.1) with 97% of sequence similarity.

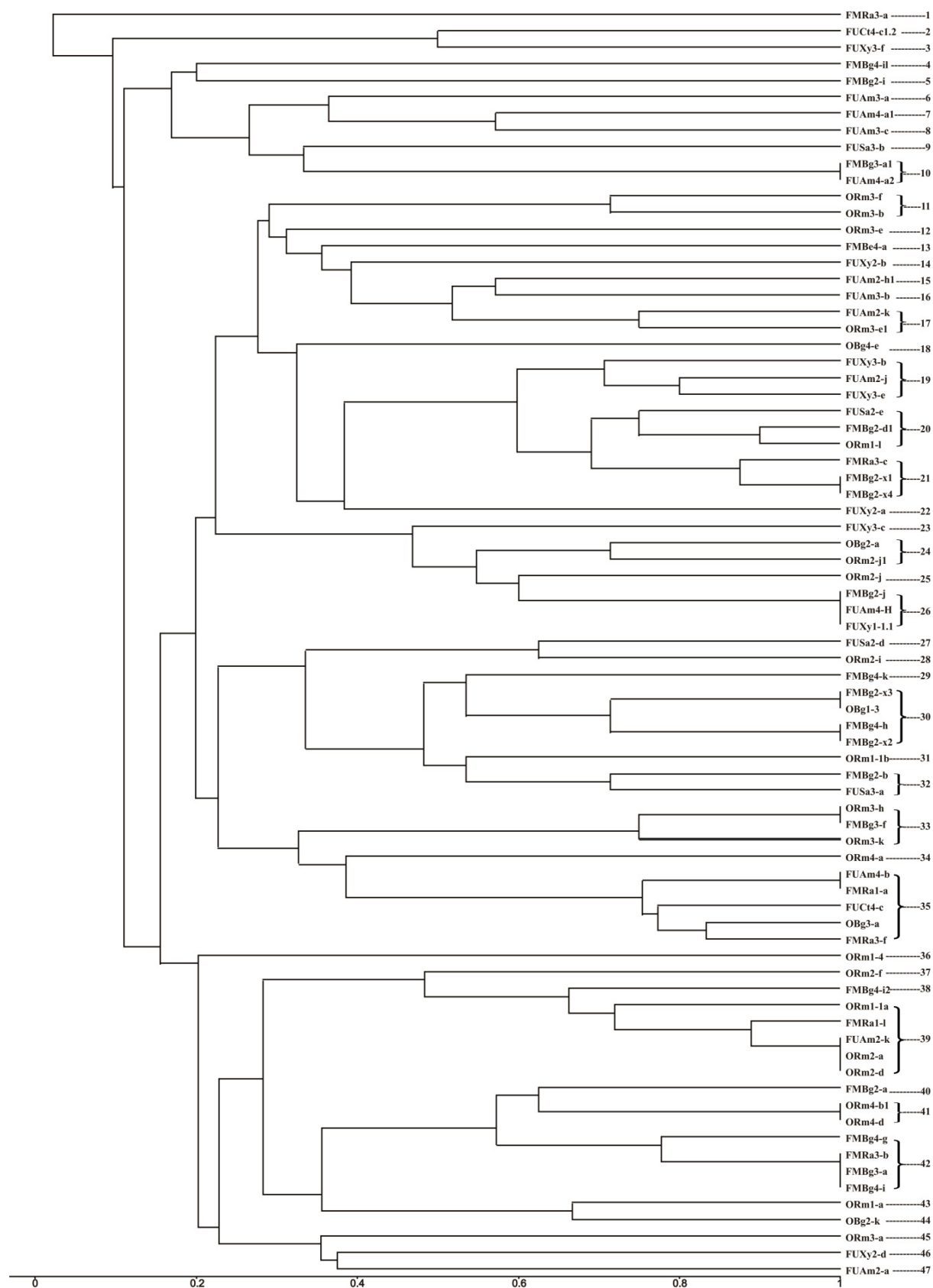
The four genus of antibiotic-producing Actinomycetes were distributed at overwash type, middle zone and upper zone of fringe type of mangrove forest of Torosiaje, Gorontalo (Figure 6). Several studies have been conducted in various mangrove ecosystems around the world and managed to discover various new types of Actinomycetes from rhizosphere of mangrove, non rhizosphere and endophytes in stems, roots and leaf of mangrove (Mangamuri et al. 2011). Several types of members of the Actinomycetes group were found in the mangrove ecosystem. They were *Streptomyces avicenniae* sp. nov., (Xiao et al. 2009), *Streptomyces qinglanensis* sp. nov., (Hu et al. 2012). The result of study show that genus *Streptomyces*, especially *Streptomyces qinglanensis* was the most dominant species and distributed across locations. Several studies were reported that the Actinomycetes community at mangrove ecosystem dominated by *Streptomyces* (Kannan and Vincent 2011; Mangamuri et al. 2014; Malek et al. 2014). *Streptomyces qinglanensis* was established by Hu et al. (2012) as a novel species of the genus *Streptomyces* that isolated from mangrove sediment at Qinglan Harbor, Wenchang, Hainan, China.



**Figure 6.** Distribution genus member of antibiotic-producing Actinomycetes at different location on mangrove forest of Torosiaje, Gorontalo

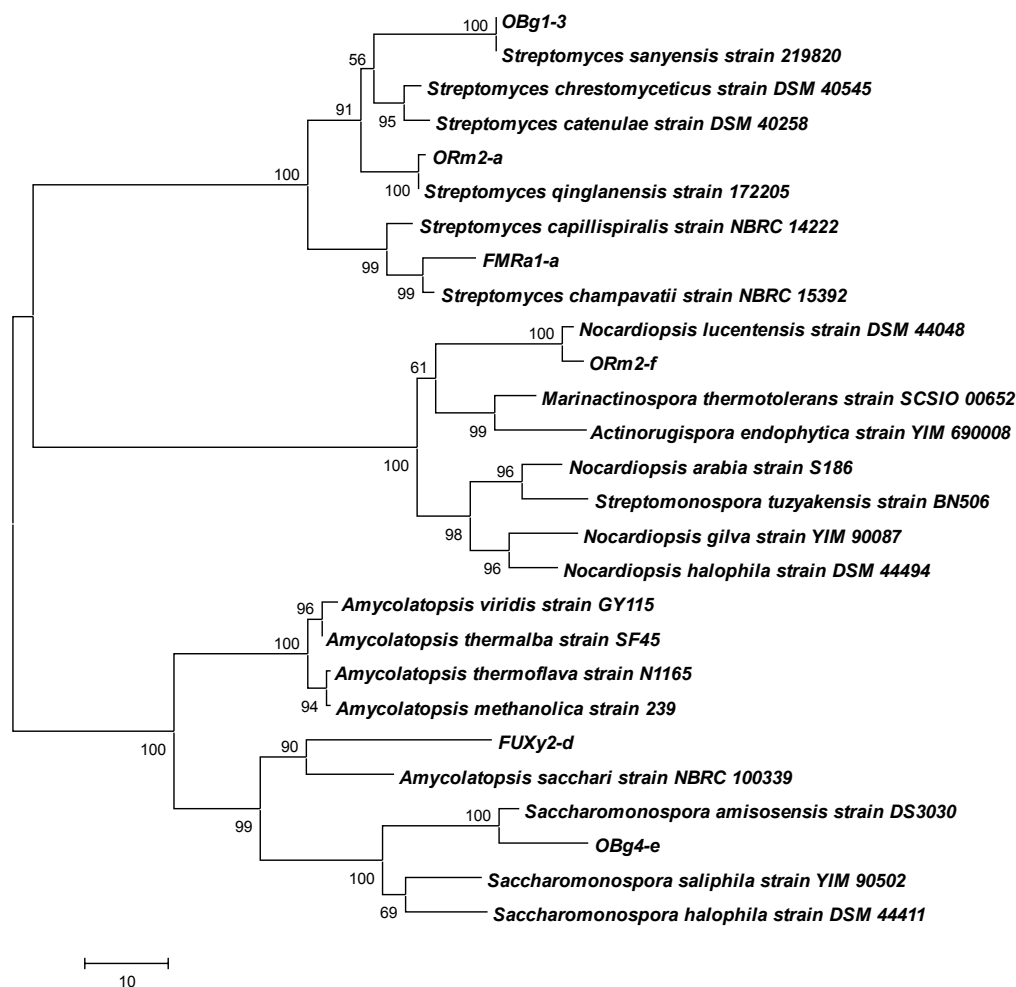
The genus *Nocardiopsis* and *Saccharomonospora* were specific found at overwash type, while genus *Amycolatopsis* at upper zone of fringe type. The genus *Saccharomonospora* which belongs to the family Pseudonocardiaceae was established by Nonomura and Ohara (1971). *Saccharomonospora amisosensis* was established by Veyisoglu et al. (2013) as novel species isolated from deep marine sediment at southern Black Sea coast of Turkey. Several members of genus *Saccharomonospora* were isolated from an extreme environments. *Nocardiopsis* strains are distributed ubiquitously in the environment. They are frequently isolated from habitats with moderate to high salt concentration such as saline soil or marine sediment (Sabry et al. 2004) and slattern (Chun et al. 2000).

In conclusion, distribution and diversity of Actinomycetes were explored from mangrove ecosystem at Torosiaje, Pohuwato District, Gorontalo Province, Indonesia. 167 actinomycete isolates were isolated from 7 types of mangrove rhizosphere. They were distributed at overwash mangrove, middle and upper zone of fringe mangrove. Among 167 actinomycete isolates, there were 77 actinomycete isolates shown antibacterial activity against pathogenic bacteria. The isolates which shown high antibacterial activity were found at rhizosphere of *A. marina* and *Xylocarpus* sp. The Actinomycetes that produce antibiotics were assigned to four genera i.e *Streptomyces*, *Saccharomonospora*, *Amycolatopsis* and *Nocardiopsis* on the basis of their 16S rDNA sequences. This is the first step towards better understanding of actinomycete from mangrove ecosystem at Torosiaje, Gorontalo.



**Figure 4.** UPGMA dendrogram inferred from ARDRA patterns of *Actinomycetes* isolated from mangrove rhizosphere. The dendrogram shows the clustering of 77 antibiotic-producing actinomycete isolates generated from amplified ribosomal DNA restriction analysis with restriction endonuclease MSP1, using the UPGMA algorithm and the Jaccard's coefficient. Number 1 to 47 represent the forty seven clusters obtained in the analysis.





**Figure 5.** Neighbour-joining phylogenetic tree inferred from 16S rRNA gene sequences. The phylogenetic tree shows the phylogenetic relationship of isolates with related genera. Bootstrap values are expressed as percentages of 1000 replications. Bootstrap values, >50% are shown at branch points. Score bar represents 1 nucleotide substitution per 100 nucleotides

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## REFERENCES

- Amrita K, Nitin J, Devi CS. 2012. Novel bioactive compounds from mangrove derived Actinomycetes. *Intl Res J Pharm* 3 (2): 25-29
- Anupa MP, Sangeetha VS, Praseetha PK, Emelda JJ, Jabaslin EE, Sugunan VS. 2013. Anticancer enzymes from Actinomycetes enhance of tumor induced mice. *J Microbiol Biotechnol Res* 3 (2): 41-50.
- Atlas RM, Bartha R. 1998. *Microbial Ecology. Fundamentals and applications*. 4th ed. Benjamin/Cummings Publishing Company, Inc. California.
- Badri DV, Vivanco JM. 2009. Regulation and function of root exudates. *Plant Cell Environ* 32: 666-681.
- Balagurunathan R, Selvan MM, Karthireshan K. 2010. Bioprospecting of mangrove rhizosphere actinomycetes from Pitchavaram with special reference to antibacterial activity. *J Pharm Res* 3 (5): 909-911.
- Basilio A, Gonzales I, Vicente MF, Gorrochategui J, Cabello A, Gonzales A, Genilloud O. 2003. Patterns of antimicrobial activities from soil actinomycetes isolated under different conditions of pH and salinity. *J App Microbiol* 95 (4): 814-823.
- Baskaran R, Vijayakumar R, Mohan PM. 2011. Enrichment method for the isolation of bioactive Actinomycetes from mangrove sediments of Andaman Islands, India. *Malays J Microbiol* 7 (1): 26-32
- Becerril-Espinosa A, Guerra-Rivas G, Ayala-Sanchez N, Soria-Mercado IE. 2012. Antitumor activity of Actinobacteria isolated in marine sediment from Todos Santos Bay, Baja California, Mexico. *Rev Biol Mar Oceanogr* 47 (2): 317-325.
- Berdy J. 2005. Bioactive Microbial Metabolites. *J Antibiot* 58 (1): 1-26
- Chaudhary HS, Soni B, Shrivastava AR, Shrivastava S. 2013. Diversity and Versatility of Actinomycetes and its Role in Antibiotic Production. *J App Pharm Sci* 3 (1): S83-S94
- Das S, Dash HR, Mangwani N, Chakraborty J, Kumari S. 2014. Understanding molecular identification and polyphasic taxonomic approach for genetic relatedness and phylogenetic relationships of microorganisms. *J Microbiol Methods* 103: 20-100.
- Doroghazi JR, Metcalf WW. 2013. Comparative genomics of actinomycetes with a focus on natural product biosynthetic genes. *Genomics* 14: 611.



- Gayatri P, Muralikrishnan V. 2013. Isolation and characterization of endophytic actinomycetes from mangrove plant for antimicrobial activity. *Intl J Curr Microbiol App Sci* 2 (11): 78-89.
- Govindasamy V, Franco CMM. 2013. Endophytic actinobacteria: diversity and ecology. In: Verma VC, Gange AC. (eds.). *Advances in Endophytic Research*, Springer, New Delhi.
- Hong K, Gao AH, Xie QY, Gao H, Zhuang L, Lin HP, Yu HP, Li J, Yao XS, Goodfellow M, and Ruan JS. 2009. Actinomycetes for marine drug discovery isolated from mangrove soils and plants in China. *Mar Drugs* 7: 24 - 44.
- Hu H, Lin HP, Xie Q, Li L, Xie XQ, and Hong K. 2012. *Streptomyces qinglanensis* sp. nov., isolated from mangrove sediment. *Int J Syst Evol Microbiol* 62: 596-600
- Hunadanamra S, Akaracharanya A, Tanasupawat S. 2013. Characterization and antimicrobial activity of *Streptomyces* strain from Thai mangrove soils. *Intl J Bioassays* 2 (5): 775-779
- Jose PA, Jebakumar SRD. 2012. Phylogenetic diversity of actinomycetes cultured from coastal multipond solar slattern in Tuticorin, India. *Aquat Biosyst* 8 (23): 1-9
- Khanna M, Solanski R, Lal R. 2011. Selective isolation of rare Actinomycetes producing novel antimicrobial compound. *Intl J Adv Biotechnol Res* 2 (3): 357-375.
- Kumar T, Ray S, Brahmachary RL, Ghose M. 2009. Preliminary GC-MS analysis of compounds present in the root exudates of three mangrove species. *Acta Chromatogr J* 21 (1). Abstract.
- Li TY, Xu F, Yan CL. 2014. Quantitatively analyzing monosaccharides in the root exudates of gray mangrove as its response to cadmium and copper stress. *WIT Trans Built Env* 156: 265-271
- Magarvey NA, Keller JM, Bernan V, Dworkin M, Sherman DH. 2004. Isolation and characterization of novel marine-derived actinomycete taxa rich in bioactive metabolites. *Appl Environ Microbiol* 70 (12): 7520 - 7529
- Malek NA, Chowdhury AJK, Zainuddin Z, Abidin ZAZ. 2014. Selective Isolation of Actinomycetes from Mangrove Forest of Pahang, Malaysia. *International Conference on Agriculture, Biology and Environmental Sciences (ICABES'14)*, 8-9 December 2014. [Bali, Indonesian]
- Mangamuri UK, Muvva V, Poda S, Kamma S. 2012. Isolation, Identification and Molecular Characterization of Rare Actinomycetes from Mangrove Ecosystem of Nizampatnam. *Malays J Microbiol* 8 (2): 83-91
- Mangamuri UK, Vijayalakshmi M, Poda S. 2014. Exploration of Actinobacteria from Mangrove Ecosystems of Nizampatnam and Coringa for Antimicrobial Compounds and Industrial Enzymes. *Br Biotechnol J* 4 (2)
- Naikpatil SV, Rathod JL. 2011. Selective isolation and antimicrobial activity of rare Actinomycetes from mangrove sediment of Karwar. *J Ecobiotechnol* 3 (10): 48-53
- Nevine BG, Soraya AS, El-sherif Zeinab M, El-ela Geha A. 2000. *J Gen Appl Microbiol* 46: 105-111
- Nolan RD, Cross T. 1988. Isolation and screening of Actinomycetes, p. 1-32. In M. Goodfellow, S.T Williams, and M. Mordarski (ed). *Actinomycetes in biotechnology*. Academic Press, San Diego, CA.
- Phongsopitana W, Suwanborirux K, Tanasupawat S. 2014. Identification and antimicrobial activity of *Streptomyces* strains from Thai mangrove sediment. *Thai J Pharm Sci* 38 (1): 49-56
- Ramirez-Elias MA, Ferrera-Cerrato R, Alarcon A, Almaraz JJ, Ramirez-Valverde G, de-Bashan LE, Ezparza-Garcia FJ. 2014. Identification of culturable microbial functional groups isolated from the rhizosphere of four species of mangrove and either biotechnological potential. *Appl Soil Ecol* 82: 1-10
- Ravikumar S, Fredimoses M, Gnanadesigan M. 2012. Anticancer property of sediment Actinomycetes against MCF-7 and MDA-MB-231 cell lines. *Asian Pac J Trop Biomed* 2 (2): 92-96.
- Ravikumar S, Ibanes SJ, Uthiraselvam M, Priya SR, Ramu A, Banerjee MB. 2011. Diversity of endophytic Actinomycetes from Karangkadu mangrove ecosystem and its antibacterial potential against bacterial pathogens. *J Pharm Res* 4 (1): 294-296
- Santhi S, Jise AA, Solomon J. 2010. Isolation and characterization of antagonistic actinomycetes from mangrove sediment. *Intl J Curr Res* 3: 20-23
- Sharma D, Kaur T, Chadha BS, Manhas RK. 2011. Antimicrobial activity of actinomycetes against multidrug resistant *Staphylococcus aureus*, *E. coli* and various other pathogens. *Trop J Pharm Res* 10 (6): 801-808.
- Suthindhiran K, Kannabiran K. 2010. Diversity and exploration of bioactive marine Actinomycetes in the Bay of Bengal of the Puducherry coast of India. *Indian J Microbiol* 50 (1): 76-82
- Waksman SA. 1950. *The Actinomycetes. Their Nature, Occurrence, Activities, and Importance*. Waltham, Mass., Chronica Botanica Company USA.
- Walker TS, Bais HP, Grotewold E, Vivanco JM. 2003. Root exudation and rhizosphere biology. *Plant Physiol* 132: 44-51
- Xiao J, Wang Y, Luo Y, Xie SJ, Ruan JS, and Xu J. 2009. *Streptomyces avicenniae* sp. nov., a novel Actinomycetes isolated from the rhizosphere of the mangrove plant *Avicennia marina*. *Intl J Syst Evol Microbiol* 59: 2624-2628.
- Xu DB, Ye WW, Han Y, Deng ZX, Hong K. 2014. Natural products from Mangrove Actinomycetes. *Mar Drugs* 12: 2590-2613.
- Zotchev SB. 2014. Genomics-Based Insights into the Evolution of Secondary Metabolite Biosynthesis in Actinomycete Bacteria in Evolutionary Biology: Genome Evolution, Speciation, Coevolution and Origin of Life. In: Pontarotti P. (ed). Springer, Switzerland.