DIVERSITY AND ANTIMICROBIAL ACTIVITY OF LICHENS-ASSOCIATED ACTINOMYCETES IN CIBINONG SCIENCE CENTRE (CSC) AND CIBODAS BOTANICAL GARDEN (CBG) INDONESIA

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Abstract

Bioprospecting has developed to all biological taxa including procaryotic. Actinomycetes become interesting procaryotic because of the ability to produce important secondary metabolite for human life. Actinomycetes are known as the largest antibiotic producer that has a broad range habitat. Some research has been done to find new antibiotic from the various habitat of actinomycetes. One of the interesting habitats of actinomycetes which never been explored in Indonesia is lichens... Lichens as the symbiotic structure of alga and fungi areknown as the ecological niche of various kinds of microorganisms including actinomycetes. Cibinong Science Centre (CSC) and Cibodas Botanical Garden (CBG) have various species of trees as the habitat of lichens. These areas are known as one of the research locations to explore the biodiversity of Indonesia. The aims of this research is to study the diversity and antimicrobial potency of actinomycetes isolated from 10 lichen samples with various type of thallus; crustose, fructose and foliose. Lichen samples were grown on the bark of 9 trees species in CSC and CBG. Isolation process used three agar media; HV, YIM6 and YIM711 with cycloheximide and nalidixic acid. Molecular identification based on 16S rRNA gene sequence. Antimicrobial activity was tested to 65 isolates by agar diffusion method to *Bacillus subtilis* BTCC B.612, *Escherichia coli* BTCC B.614, *Candida albicans* BTCC Y.33, *Staphylococcus aureus* BTCC B.611, *Micrococcus luteus* BTCC B.552. Isolation process retrieved 125 isolates with the highest number grow on HV agar medium. Based on the sample, the highest number of actinomycetes were isolated from crustose lichen attached on the bark of *Averrhoea carambola*. A total 69 isolates were identified as the genera Actinoplanes, Amycolatopsis, Angustibacter, Kribbella, Micromonospora, Mycobacterium, and Streptomyces. The screening process showed 24 isolates have antimicrobial activity, with the highest inhibitory activity against *Micrococcus luteus* BTCC B.552.

Keywords: Actinomycetes, Antimicrobial activity, Diversity, Identification 16S rRNA, Lichens

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Introduction

The pharmaceutical industry was implication of the bioprospecting development in humans life. Exploration various organisms as the drug sources has been developed to all biological taxa including procaryotic (Parrot *et al.*, 2015). Ability of prokaryotic produce metabolite to inhibit the growth of pathogen microbes then explored as the source of new drugs. The discovery of new drugs in academics and laboratory level has traditionaly been focused on the exploitation of actinomycetes and filamentous fungi (Genniloud *et al.,* 2011).

Actinomycetes are virtually unlimited source of novel compound with many therapeutic application and hold a prominent position due to their diversity and ability to produce novel bioactive compound

DOI: http://dx.doi.org/10.14203/ann.bogor.2019.v22.n2.1-12

(Subramani and Aalbersberg, 2012). These Gram-positive bacteria were reported able to produce antibacterial (Lazzarini *et al.,* 2000; Liu *et al.,* 2017), anticandidal (Charausova *et al.,* 2016), antiparasitic (Pimentel-Elardo *et al.,* 2010) and antiviral (Sacramento *et al.,* 2004). The ability of actinomycetes as the highest producer of antibiotics from prokaryotes has been known for decades (Berdy, 2012). Two third of the world's antibiotics including the most important in medical treatment produce by actinomycetes in the genus of Streptomyces and Micromonospora (Kumar *et al.,* 2010). This fact become one reason the research about actinomycetes keep doing recently to find new antibiotics compound.

Actinomycetes has the widest distribution among other bacteria in nature (Kumar *et al.,* . 2010). It's broad habitat urged some research to find new compound through exploration of ecological niches that still rare to be explored. Invention of new source of actinomycetes being a good choice for pharmaciteucal development (Jiang *et al.,* 2016). Some research reported successfully isolated actinomycetes from various rare habitat such as endosymbiont of plants (Nimnoi *et al.,* 2009), extreme environmental (Tang *et al.,* 2002; Okoro *et al.,* 2009), and symbiosis of microorganisms such as lichens (Jiang *et al.,* 2015; Parrot *et al.,* 2015; Liu *et al.,* 2017).

One source that interesting to be explored is lichens. Lichens are the symbiotic of alga/cyanobacteria and fungi (Liu *et al.,* 2017). Even it is less commonly that lichens involved three or more partners (Nash, 2008). The structure of this organisms made it possible to produce thousands of bioactive compound (Jiang *et al.,* 2015). Lichens also being habitat for various bacteria, that the roles in lytic activity was important to produce bioactive compound such as hormons or antibiotics to fulfill nitrogen requirement (Grube, 2009).

Research about diversity of lichens-associated actinomycetes confirmed that actinomycetes from tropic lichens more diverse than cold area (Gonzales *et al.,* 2005). This report recently encourages studies about lichens-associated actinomycetes in some Asian countries such as Japan (Hamada *et al.,* 2012) and China (Jiang *et al.,* 2015; Liu *et al.,* 2017). New species of actinomycetes was isolated from lichens such as *Nocardioides exalbidus* sp. nov. (Li *et al.,* 2007) and *Luteimicrobium album* sp. nov. (Hamada *et al.,* 2012). Some actinomycetes isolated from lichens also reported has ability to inhibit the growth of another microorganisms and being potencial source for novel drugs development (Jiang *et al.,* 2015).

Indonesia as one of tropical country has a very diverse wooden trees as the lichenes habitat. However, the study about lichen-associated actinomycetes from Indonesian was not reported. Therefore the aim of this study was to analyze the diversity and antimicrobial potency of lichen-associated actinomycetes from Indonesia. Some representative area with diverse wooden trees as lichens habitat located in west java; Cibinong Science Centre (CSC) which have 901 trees species (Noviady and Rivai 2015) and Cibodas Botanical Garden (CBG) which have 3,120 trees species (Rosyunita, 2017). This fact promising high diversity of actinomycetes that hopefully can be new source for potential antimicrobial compound to enrich bioprospecting in pharmaceutical.

Materials and Methods

Study Area and Sampling of tree lichens

This study was conducted from October 2017 to July 2018. Samples of lichens were various in thallus type and collected aseptically from the bark of tree in West Java Indonesia. Sampling sites were located in Cibinong Science Centre (CSC) with coordinate S. 6°29'49.7"; E. 106°50'45.3" and Cibodas Botanical Garden (CBG) with coordinate S. 6°44'22.1"; E. 107°00'24.9". Total 5 samples of lichens were taken from 4 tree species in CSC; *Cynometra cauliflora, Gnetum gnemon, Averrhoa carambola, Artocarpus integra* (Figure 1a). Another 5 samples were taken from 5 tree species in CBG*; Pandanus utilis, Magnolia* sp., *Brachychiton sp., Tristahiopsis taurina,* and *Cupresson torolusa* (Figure 1b). Lichens samples were taken by sterile cutter and collected in sterile plastic bag. The rest process for this study was conducted in the Laboratory for Applied Microbiology, Indonesian Institute of Sciences.

Isolation of Lichen-Associated Actinomycetes

Each lichens sample was put in sterile microtubes. Each lichen samples (0.1 g) was put in the steril microtube, added by 1ml aquadest, then homogenized 3,000 rpm for 1 minute. The sample was centrifuged in

DOI: http://dx.doi.org/10.14203/ann.bogor.2019.v22.n2.1-12

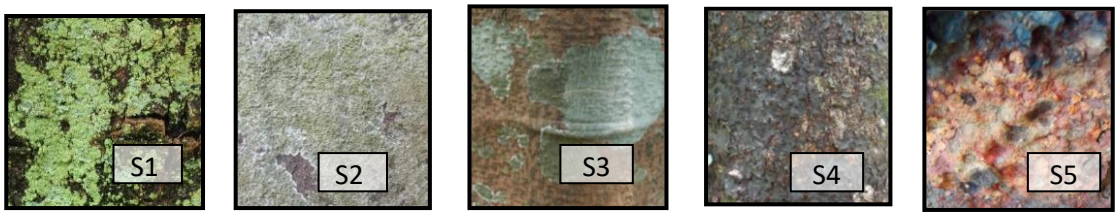

Figure 1a. Lichens from Cibinong Science Centre (CSC); S1: bark of *Cynometra cauliflora*, S2: bark of *Gnetum gnemon*, S3: bark of *Averrhoa carambola*, S4 and S5: bark of *Artocarpus integra*

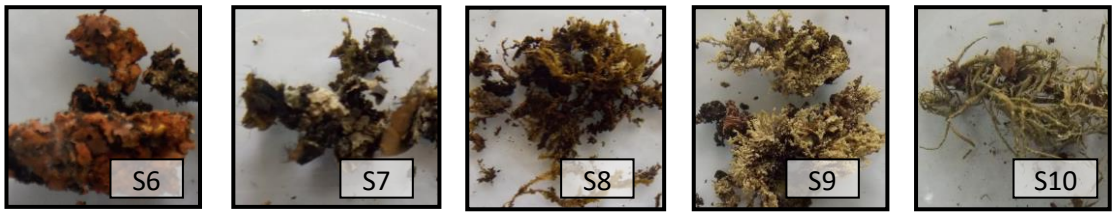

Figure 1b. Lichens from Cibodas Botanical Garden (CBG); S6: bark of *Pandanus utilis*, S7: *Magnolia sp.*, S8: bark of *Brachychiton sp.*, S9: bark of *Tristahiopsis taurina*, and S10: bark of *Cupresson torolusa*

13,000rpm for 5 minutes, the supernatan was then throwed out. This treatment was repeated for three times. Lichens was mashed in the sterile plastic with addition of 1 ml aquadest. Mashed lichen (1 ml) was mixed with 9 ml aquadest as the first dilution. About 1 ml from first dilution added with 9 ml aquadest as the second dilution. Dilution process was done until 10^{-5}. About 100 µl dilution mixture was then spread to each plate of isolation medium. This study was conduct with three kind of agar medium for isolation with the following ingredients per liter. HV agar: Humic acid 1 g, Na2HPO4 0.5 g, KCl 1.7 g, MgSO4.7H2O 0.05 g, FeSO4.7H2O 0.01g, CaCl2 1 g, B-vitamins (0.5 mg each of thiamine-HCl, riboflavin, Niacin, pyridoxin, Capantothenate, inositol, p-aminobenzoic acid, and 0.25mg of biotin), agar 18 g, water 1000 ml, pH 7.4 (Hayakawa, 2008).

YIM 6: soluble starch 10g, casein 0.3g, KNO3 2g, MgSO4•7H2O 0.05g, NaCl 2g, K2HPO4 2g, CaCO3 0.02g, FeSO4 10mg, Vit mixture of HV medium 3.7mg, agar 15g. pH 7.2. YIM 711: Casein 1.5g, soybean peptone 0.5g, K2HPO4•H2O 1g, MgSO4 •7H2O 0.5g, CaCO3 0.3g, NaCl 5g, Vit mixture of HV medium 3.7mg, agar 15g. pH 7.5 (Jiang *et al.*, 2015).

All the medium added by 50 mg cycloheximide and 50 mg as fungi inhibitor and 40 mg nalidixic acid as the Gram-negative bacteria inhibitor (in liter). Lichens samples from CSC was diluted for 10^{-3}, 10^{-4}, 10^{-5}. While samples from CBG used dilution 10-1, 10^{-2} to coat YIM 6 and YIM 711, dilution 10^{-2}, 10^{-3} on HV agar media. Cultivation was done in 30 ºC for 7-21 days. The colony was observed under the microscope with 10 times magnificient. A single actinomycetes colony was isolated to yeast extract-malt extract agar (ISP-2) medium. The pure strain were conserved in 20% of glycerol at -80 ºC (Jiang *et al.*, 2015; Liu *et al.*, 2017).

Extraction of Genomic DNA

One ose pure strain on ISP-2 agar was cultured into 5 Tryptic Soy Broth (TSB) (Axenov-Gibanov, 2016). About 1 ml culture was put into microtube and centrifuged on 13000 rpm for 5 minutes. The supernatan was throw out, and the sedimen used for DNA extraction. DNA pellet washed by TE-buffer then centrifuged at 13,000 rpm for 5 minutes, this step was done twice. The cleaned sedimen added by 300 µl buffer extraction added to, mashed the pellet then warm up in 60 ºC water bath for 10 minutes. The sedimen was then cold in room temperature 25 ºC and added 150 µl sodium asetat. The mixture was put in room temperature for 10 minutes and centrifuged 13,000 rpm for 5 minutes. Supernatan was taken and added by isopropanol with ratio 1:1, centrifuged 13,000 for 10 minutes to get DNA extract. The DNA washed by 500 µl etanol and added by TE buffer 50 µl (Ratnakomala, 2016).

Amplification of 16S rRNA

The 16SrRNA gene was amplified by mixed 1 µL isolate DNA in measured concentration, with 2 µL PCR mixture that

DOI: http://dx.doi.org/10.14203/ann.bogor.2019.v22.n2.1-12

consists of HS ready mix, ddH_2O, and forward primer 9F and reverse 1541R 9F (forward: 5'GAGTTTGATCCTGGCTCAG-3' position 9-27) and 1541R (Reverse: 5'-AAGGAGGTG ATCCAGCC3' position 1541-1525) with concentration 20 pmol. Thermal cycling using the following procedure: Denaturation 96^{o}C for 5 minutes, followed by 30 cycles denaturation 96 ^{o}C for 30 seconds, annealing at 55 ^{o}C for 30 seconds, extension at 72 ^{o}C for 7 minutes (Widyastuti and Ando 2010). Visualization PCR product on agarose 1%, under UV transilluminator. The PCR product then used in sequenceing process using BigDye Terminator Version 3.1 Cycle Sequencing Kit. Sequencing Product analyzed by DNA Sequencer ABI Prism 3700 (Applied Biosystem) by the factory protocol. Sequencing product edited using bioedit and confirmed online by ez-Taxon server. Phylogenetic trees based on the 16S rRNA (Kim *et al.,* 2012) gene sequences were generated using the neighbor-joining method from the software package MEGAversion 6.0 (Tamura *et al.,* 2013).

Determination Antimicrobial Activity

The pure strains on ISP-2 agar was screened by agar diffusion method against bacteria and yeast to determined it's antimicrobial potency. The microbial that were used as indicator for the test consist of three Gram positives bacteria: *Bacillus subtilis* BTCC B-612, *Micrococcus luteus* BTCC B-552, *Streptococcus aureus* BTCC B-611, Gram negative bacteria *Escherichia coli* BTCC B-614 and fungi *Candida albicans* BTCC Y-33. The tested bacterial were cultured in Nutrient Broth (NB) and Candida cultured in Potato Dextrose Broth (PDB) medium for 24 hours. The 9 mm pure strains of actinomycetes was taken put on the surface of soft layer agar after drain up, and incubated in optimum temperature of each microbial tested for 18-24 hours (Ratnakomala *et al.,* 2016a). The inhibition zone was measured in scale.

Results

Lichen sample

This research used lichens as the source of actinomycetes. Lichens are the symbiotic organisms, usually composed fungal as mycobiont and one or more photosynthetic partner such as green alga or cyanobacterium as photobiont (Nash, 2008). Lichen-associated microbial communities consist of diverse taxonomic in group. Various bacterial genera found in lichens led to speculation of their different role to support life of lichens. Bacteria can be involved in defense against lichens pathogens and feeders. Davies *et al.,* . 2005 reported that lichen-associated actinomycetes showing to be potent antibiotics at very low concentration. This result also suggests that low-abundance strain could play significant roles in the lichen micro-ecosystem (Grube and Berg, 2009).

Lichens sample in this research has three kinds of thallus type; crustose, fruticose, and foliose. Based on Hale (1979), sample 1, 2, 3, 5 and 6 belong to crustose lichens. These lichens have a fairly thick thallus but the margins are unlobed and sometimes fade into the substrate and become indistinct. Removal process from the bark must destroy the thallus. Sample 8, 9, and 10 belong to fruticose lichens, with bushy and hairy thallus attached to the tree bark. Sample 4 and 7 belongs to foliose lichen, the thallus growth outward from the and becoming round at outline part.

Medium and Isolation Effectiveness

Isolation was conducted for 10 lichens sample from 9 trees from area of Cibinong Science Centre (CSC) and Cibodas Botanical Garden (CBG) West Java with spread dilution method. Sample 1 to 5 were from CSC area (in blue graph), while sample 6 to 10 were from CBG (in red graph). Dilution for lichens from CSC was conducted from 10^{-3} until 10^{-5}, and samples from CBG used dilution 10^{-1} until 10^{-3}. Totally 125 isolates were collected from the isolation process. Each sample resulted various numbers of isolates (Figure 2). Total actinomycetes isolated from CSC sample was 97 isolates and from CBG sample was 28 isolates.

Most isolates resulted from sample 3, crustose lichen from the bark of *Averhoea carambolla* (32 isolates) followed by sample 5 crustose lichen from the bark of *Artocarpus integra* (26 isolates) and sample 8 fruticose from the bark of *Brachychiton* sp. (21 isolates). A 62% isolates resulted from crustose lichen, since most of samples (5 of 10) has crustose thallus. Compare to research conducted by Liu *et al.,* (2017), most isolates

DOI: http://dx.doi.org/10.14203/ann.bogor.2019.v22.n2.1-12

(67%) resulted from foliose lichen. This research used 23 foliose from 35 lichen sample. Both research showed that the isolates number didn't affected by type of lichen as the samples.

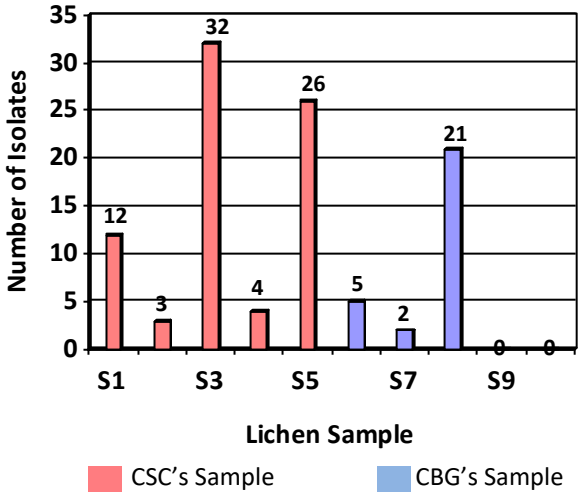

Figure 2. Number of Isolates Based on Lichens Samples

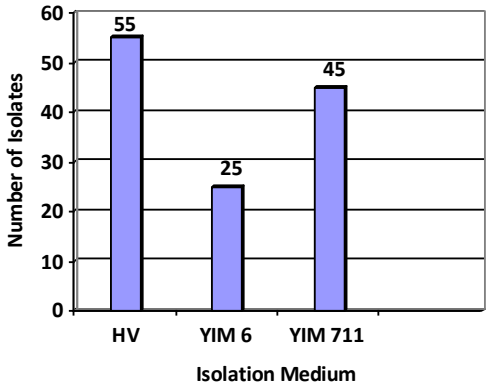

Figure 3. Number of Selected Isolates Based on Morphological character

Three kinds of agar medium were used in isolation process; HV, YIM 6, and YIM 711 medium. Starch-casein medium (YIM 6) and Casein Soybean peptone medium (YIM 711) was designed as the isolation medium foractinomycetes from several habitats. One kind of actinomycetes that can use YIM 6 and YIM 711 was lichens-associated actinomycetes. The design of YIM 6 and YIM 711 for lichen-associated actinomycetes isolation proces based on some factors, such as isolation goals, medium component, and inhibitors. The component (carbon and nitrogen sources) of selective isolation media was formulated by using information from taxonomic databases and phenotypic databases of actinomycetes as the isolation target (Jiang *et al.*, 2016).

The Humic acid-Vitamins (HV) agar contain soil humic acid as carbon and nitrogen source. The humic reserve of soils are thought to represent several times the total organic carbon in living organisms; and more than half of the total organic carbon in soils. The occurence of carboxyl and hidroxyl groups on the periphery of humic macro molecules plays a major roles by facilitating the formation of mineral ions or small organic molecules. Humic acid generally are resistant to biological decomposition. However, actinomycetes have been shown capable of utilizing the humic acid. Its implicate HV agar as the efficient and adequate medium of growth for *Streptomycetes* and various rare-actinomycetes, while restricting growth of non-filamentous bacteria colonies (Dari *et al.*, 2008; Hayakawa, 2008).

Diversity of Actinomycetes from lichen

A 125 isolates of actinomycetes resulted from 10 lichen samples. Morphological observation showed most of isolates posses the *Streptomyces* genera with characteristics; slow growing, aerobic, glabrous, or chalky, heaped, folded and have different collor of aerial and substrat mycelia. Some of isolates with the certain character also resulted earthy oddor (Suneetha *et al.*, 2011).

Contamination by fungi became the problem in the isolation process, so that only 69 isolates can be molecularly identified and 65 isolates were screened for the antimicrobial activities. The 69 isolates of actinomycetes were identified based on 16S rRNA and confirmed to ez-Taxon to closest species and genus level (Table 1). Cultivation of isolates in in Tryptic Soy Broth (TSB) was conducted to get DNA extract of actinomycetes. Molecular identification showed that 69 isolates belong to 7 genera; *Actinoplanes*, *Amycolatopsis*, *Angustibacter*, *Kribbella*, *Micromonospora*, *Mycobacterium*, and *Streptomyces*. The 87% of isolates belongs to *Streptomyces* with 23 closests species, followed by 4.3% *Micromonospora*, 2.9% *Kribbella* and each 1.4% for *Actinoplanes*, *Amycolatopsis* and *Mycobacterium*. All identified genera also found in lichen from Yunan provinces China (Jiang *et al.*, 2015; Liu *et al.*, 2017). Based on molecular identification, most genera isolated

DOI: http://dx.doi.org/10.14203/ann.bogor.2019.v22.n2.1-12

from Cibinong Science Centre (CSC) and Cibodas Botanical Garden (CBG) belongs to *Streptomyces*. This genera successfully isolated using all kind of agar medium in this research. *Streptomyces* was the dominant genus consits of almost 600 species (Kampfer *et al.*, 2008). Actinoplanes, Angustibacter, and Mycobacterium were isolated using HV. Kribbella and Angustibacter were isolated using YIM 6, while Mycobacterium was isolated using YIM 711.

Table 1. Identification of Lichens-Associated Actinomycetes based on 16S rRNA gene similarity

No	Isolate	Genus	Scientific Name	Lichen Sample	BLAST Identity
1	LC-1	*Micromonospora*	*Micromonospora chersina*	S1	1388/1389 (99,93%)
2	LC-2	*Streptomyces*	*Streptomyces seoulensis*	S1	1413/1416 (99,79%)
3	L C-3	*Streptomyces*	*Streptomyces violacerubidus*	S1	1418/1437 (98,66%)
4	LC-4	*Streptomyces*	*Streptomyces kunmingensis*	S1	1419/1428 (99,36%)
5	LC-5	*Actinoplanes*	*Actinoplanes couchii*	S1	1370/1371 (98,90%)
6	LC-6	*Streptomyces*	*Streptomyces kunmingensis*	S1	1089/1095 (99,45%)
7	LC-8	*Streptomyces*	*Streptomyces kunmingensis*	S1	1407/1415 (99,43%)
8	LC-9	*Streptomyces*	*Streptomyces kunmingensis*	S1	1406/1416 (99,29%)
9	LC-10	*Streptomyces*	*Streptomyces thermoviolaceous*	S1	1404/1426 (98,43%)
10	LC-11	*Micromonospora*	*Micromonospora schwarzwaldensis*	S2	1415/1422 (99,50%)
11	LC-13	*Micromonospora*	*Micromonospora schwarzwaldensis*	S2	1404/1410 (99,57%)
12	LC-14	*Streptomyces*	*Streptomyces seoulensis*	S3	1412/1413 (99,93%)
13	LC-15	*Streptomyces*	*Streptomyces cinerochromogenes*	S3	1414/1429 (98,94%)
14	LC-16	*Streptomyces*	*Streptomyces seoulensis*	S3	1464/1613 (90,72%)
15	LC-17	*Angustibacter*	*Angustibacter luteus*	S3	1414/1428 (99,01%)
16	LC-19	*Streptomyces*	*Streptomyces cinerochromogenes*	S3	1410/1428 (98,72%)
17	LC-21	*Streptomyces*	*Streptomyces similanensis*	S3	1476/1507 (97,94%)
18	LC-22	*Streptomyces*	*Streptomyces collinus*	S3	1409/1420 (99,22%)
19	LC-23	*Streptomyces*	*Streptomyces palmae*	S3	1408/1429 (98,51%)
20	LC-25	*Streptomyces*	*Streptomyces collinus*	S3	1411/1421 (99,29%)
21	LC-26	*Streptomyces*	*Streptomyces seoulensis*	S3	1403/1422 (98,65%)
22	LC-27	*Streptomyces*	*Streptomyces seoulensis*	S3	1411/1413 (99,86%)
23	LC-28	*Streptomyces*	*Streptomyces thermoviolaceus*	S3	1410/1412 (99,86%)
24	LC-31	*Streptomyces*	*Streptomyces cinerochromogenes*	S3	1430/1445 (98,94%)
25	LC-35	*Streptomyces*	*Streptomyces rochei*	S3	1418/1428 (99.30%)
26	LC-36	*Streptomyces*	*Streptomyces badius*	S3	1411/1413 (99.86%)
27	LC-37	*Streptomyces*	*Streptomyces palmae*	S3	1434/1453 (98.67%)
28	LC-40	*Streptomyces*	*Streptomyces collinus*	S3	1423/1432 (99.37%)
29	LC-41	*Streptomyces*	*Streptomyces lomordensis*	S4	1426/1433 (99.51%)
30	LC-44	*Streptomyces*	*Streptomyces roseolus*	S4	1403/1412 (99.36%)
31	LC-47	*Streptomyces*	*Streptomyces cinereoruber*	S4	1414/1419 (99.65%)
32	LC-50	*Kribella*	*Kribella aluminosa*	S4	1394/1407 (99.07%)
33	LC-51	*Streptomyces*	*Streptomyces atriruber*	S4	1414/1424(99.22%)
34	LC-52	*Streptomyces*	*Streptomyces atriruber*	S4	1424/1436 (99.15%)
35	LC-56	*Kribella*	*Kribella karoonensis*	S4	1422/1441 (98.65%)
36	LC-57	*Streptomyces*	*Streptomyces roseolus*	S4	1419/1428 (99.36%)
37	LC-58	*Streptomyces*	*Streptomyces puniceus*	S4	1424/1425 (99.93%)
38	LC-60	*Streptomyces*	*Streptomyces puniceus*	S4	1448/1449 (99.93%)
39	LC-66	*Streptomyces*	*Streptomyces puniceus*	S5	1424/1425 (99.93%)
40	LC-67	*Streptomyces*	*Streptomyces seoulensis*	S5	1426/1427 (99.93%)
41	LC-68	*Streptomyces*	*Streptomyces violacerubdius*	S5	1421/1441 (98.58%)
42	LC-69	*Streptomyces*	*Streptomyces coerulescens*	S5	1407/1411 (99.50%)
43	LC-71	*Streptomyces*	*Streptomyces althioticus*	S5	1421/1423 (99.86%)
44	LC-73	*Streptomyces*	*Streptomyces althioticus*	S5	1430/1432 (99.86%)
45	LC-74	*Streptomyces*	*Streptomycesrhizosphaerihabitans*	S5	1429/1439 (99.30%)
46	LC-76	*Amycolatopsis*	*Amycolaptosis rubida*	S5	1412/1430 (98.72%)
47	LC-77	*Streptomyces*	*Streptomyces seoulensis*	S5	1428/1431 (99.79%)
48	LC-78	*Streptomyces*	*Streptomyces palmae*	S5	1421/1441 (98.59%)
49	LC-79	*Streptomyces*	*Streptomyces seoulensis*	S5	1412/1415(99.79%)
50	LC-80	*Streptomyces*	*Streptomyces violacerobidus*	S5	1426/1446 (98.59%)
51	LC-81	*Streptomyces*	*Streptomyces violaceorectus*	S5	1424/1433(99.37%)
52	LC-82	*Streptomyces*	*Streptomyces fragilis*	S5	1414/1421(99.50%)
53	LC-83	*Streptomyces*	*Streptomyces collinus*	S5	1409/1418 (99.36%)
54	LC-84	*Streptomyces*	*Streptomyces collinus*	S5	1410/1420 (99.29%)
55	LC-86	*Streptomyces*	*Streptomyces seoulensis*	S5	141/1413(99.86%)

DOI: http://dx.doi.org/10.14203/ann.bogor.2019.v22.n2.1-12

56	LC-87	*Streptomyces*	*Streptomyces aureus*	S6	14181419 (99.89%)
57	LC-88	*Streptomyces*	*Streptomyces collinus*	S5	1413/1422 (99.36%)
58	LC-89	*Streptomyces*	*Streptomyces seoulensis*	S6	1427/1428 (99.93%)
59	LC-94	*Streptomyces*	*Streptomyces caniferus*	S8	1409/1411 (99.86%)
60	LC-96	*Streptomyces*	*Streptomyces seoulensis*	S8	1418/1420 (99.86%)
61	LC-100	*Streptomyces*	*Streptomyces camponoticapitis*	S8	1389/1394 (99.64%)
62	LC-103	*Streptomyces*	*Streptomyces aureus*	S8	1426/1428 (99.86%)
63	LC-109	*Streptomyces*	*Streptomyces collinus*	S8	1418/1427 (99.36%)
64	LC-110	*Streptomyces*	*Streptomyces seoulensis*	S8	1416/1417 (99.93%
	Continue..				
65	LC-111	*Streptomyces*	*Streptomyces seoulensis*	S5	1407/1408 (99.93%)
66	LC-112	*Streptomyces*	*Streptomyces cinerochromogenes*	S3	1422/1439 (98.80%)
67	LC-118	*Streptomyces*	*Streptomyces palmae*	S3	1433/1427 (97.32%)
68	LC-122	*Mycobacterium*	*Micobacterium neworleanense*	S8	1434/1453 (98.66%)
69	LC-125	*Streptomyces*	*Streptomyces kunmingensis*	S1	1416/1423 (99.50%)

Based on data in Table 1, each lichen samples resulted various genus and species. Sample 1, the crustose lichen from the bark of *Cynometra cauliflora* has the most diverse genus of actinomycetes. Three genus were isolated from this lichen; *Actinoplanes*, *Micromonospora*, and *Streptomyces*. Two genus; *Angustibacter* and *Streptomyces* were isolated from sample 3, the crustose lichens of Averhoea carambola. Genus *Kribbella* and *Streptomyces* isolated from sample 4, foliose lichens of Artocarpus integra. Genus *Amicolaptosis* and Streptomyces isolated from sample 5, crustose lichen of *Artocarpus integra*. Genus *Mycobacterium* and *Streptomyces* isolated from sample 8, fruticose lichen of *Brachychiton* sp. Sample 2 and 6 consists of only one genus.

The most diverse species of actinomycetes belongs to sample 5, crustose lichens of *Artocarpus integra*. This lichen consist of 10 closest species. This result followed by sample 3 (crustose lichens of *Averhoea carambola*) and sample 4 (foliose lichens of *Artocarpus integra*) with 9 and 8 species of each. This result showed that the most diverse actinomycetes did not belong to any kind of lichen thallus.

Antimicrobial Activity of Lichens Associated Actinomycetes

Determination of antimicrobial activity was conducted by agar plug diffusion method toward 65 isolates. Antimicrobial activity showed by 24 isolates to at least one tested microbial (Table 2). Fifteen isolates were able to inhibit the growth of *Bacillus subtilis* and *Micrococcus luteus*, and 6 isolates were inhibit *Staphylococcus aureus*. Two isolates were able to inhibit the growth of *Candida albicans*, and only one isolate inhibited the growth of *Escherichia coli*. Most inhibition zones were formed against Gram-positive bacteria; *Bacillus subtilis*, *Micrococcus luteus* and *Staphylococcus aureus* compare to Gram-negative bacteria (*Escherichia coli*). This result because the differences in cell wall constituent and arrangement between Gram-positive and Gram-negative bacteria. Gram positive bacteria cell walls contain peptidoglycan layer, an ineffective permeability barrier (Pratiwi, *et al.*, 2016). The outer membrane of Gram negative bacteria contain lipopolysaccharide component an effective barrier against hydrophobic substances.Antimicrobial activity against Candida albicans as Eukaryotes cell also lower than Gram-positive bacteria, because organelles of this organism protected by membrane-enclosed nucleus and DNA that make the cell structucally more complex than prokaryotes (Madigan *et al.*, 2012).

The isolates LC-23, LC-94, and LC-100 (figure 4) screening result showed were able to inhibit at least three microbial tested. Isolate LC-23 able to inhibit all of Gram-positive bacteria. It has potency as anti Gram-positive bacteria. Isolate LC-94 was able to inhibit all Gram-positive bacteria and fungi *Candida albicans*. It was potential as anti Gram-positive bacteria and anti-candida. Isolates LC-100 was able to inhibit the growth of all bacteria belongs to Gram positive and Gram negative. This isolate was potential as broad spectrum antibiotics.

DOI: http://dx.doi.org/10.14203/ann.bogor.2019.v22.n2.1-12

Table 2. Inhibition zone diameters of 24 isolates of lichen-associated Actinomycetes (coloni diameters around 5 mm) against tested microorganisms.

No	Isolates	Diameter Inhibition Zone (mm)				
		B. subtilis	*M. luteus*	*S. aureus*	*E. coli*	*C. albicans*
1	LC-2	16,0	20.0			
2	LC-6		12.0			
3	LC-8	17.0	25.0			
4	LC-15	11.2				
5	LC-23	21.0	23.0	22.5		
6	LC-28	11.0				
7	LC-29			12.7		
8	LC-32			11.5		
9	LC-36	13.0				
10	LC-37	20.0	16.0			
11	LC-41	10.0				
12	LC-46		23.0			
13	LC-49	10.0	19.0			
14	LC-51	8.0	20.0			
15	LC-52		19.0			
16	LC-58	10.0	11.0			
17	LC-74					10.8
18	LC-75	9.0				
19	LC-84			12.0		
20	LC-86		8.55			
21	LC-94	8.8	8.5	10.0		14.3
22	LC-100	22.8	26.8	23.3	17.4	
23	LC-105		8.5			
24	LC-115	20.7	19.0			
	Total isolat	15	15	6	1	2

Characteristic of Isolate LC-23, LC-94, and LC-100

Sequence data of isolates LC-23, LC-94, and LC-100 has been submited to gene bank of NCBI. The accession number for sequence LC-23 was SUB5611109 LC-23 MK910204, accession number for sequence LC-94 was SUB5610932 Streptomyces.MK910159, and accession number for sequence LC-100 was SUB5595872 Streptomyces MK898926.

Molecular identification of 16S rRNA gene conducted to the three isolates showed that LC-23 has 98.51% similarity to *Streptomyces palmae* isolated from rhizosphere of oil palm (*Elaeis guineensis* Jacq.). This closests *Streptomyces* has 21 different bases over 1408 total basephare analyze. *Streptomyces palmae* reported has grey to light brown mycelium color and showed antifungal activity (Sujarit *et al.*, 2016). Isolates LC-23 showed white aerial mycelium and able to change ISP 2 medium into yellow. Isolate LC-94 has 99.86% similarity to *Streptomyces caniferus* with difference 2 bases over 1409 base analysed. *Streptomyces caniferus* has white color in ISP 2 and grey in ISP 3 to ISP 7 (Strain, 1986), it used to be isolated from polychaeta *Filograna* sp.and has antifungal against *Candida albicans* and cytotoxic activity against A549 human lung carcinoma cells, MDA-MB-231 human breast adenocarcinoma cells and HT29 human colorectal carcinoma cells (Perez *et al.*, 2016). Isolate LC-94 also has antifungal activity against *Candida* albicans, showed grey color on ISP 2 and not able to change medium color. Isolates LC-100 has 99.64% similarity to *Streptomyces camponoticapitis* with 5 bases difference over 1388 base analyzed. *Streptomyces camponoticapitis* isolated from the head of ant *Camponotus japonicus* Mayr showed varies mycelium from colorless to moderate yellow (Li *et al.*, 2016). Isolate LC-100 showed white aerial mycelium on ISP 2 and not able to change the medium color.

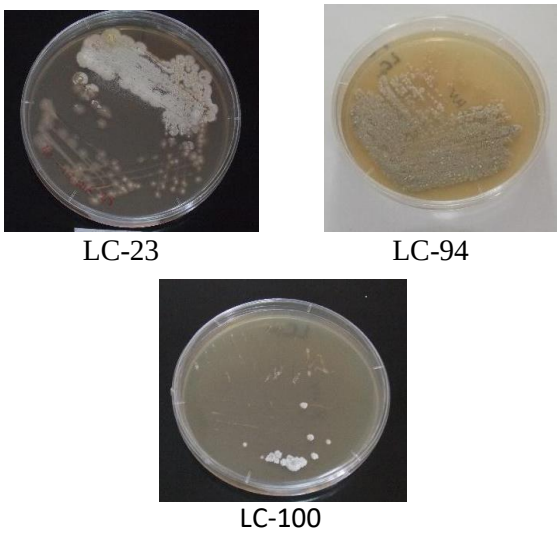

Figure 4. Top Surface Appearence of the isolate LC-23, LC-94, and LC-100 on ISP 2 Agar Medium

Neighbor joining method of phylogenetic tree analyze was constructed to explain taxonomical position of isolates LC-23, LC-94, and LC-100 compared to their type strain in genus *Streptomyces* (Figure 5). Sequence of *Aquifex phyrophlus* Ko15a used as the outgroup in the phylogenetic tree. Numbers at nodes are bootstrap values based on 1000 resamplings.

Result analyse of phylogenetic tree showed that the closest species of tree isolate differ from 16S rRNA analyze. Closest species of isolate LC-94 was *Streptomyces glebosus* with the similar value 99.78%, while for isolate LC-100 the closest species was *Streptomyces*

DOI: http://dx.doi.org/10.14203/ann.bogor.2019.v22.n2.1-12

niveus with similar value 99.56%. The interesting result showed by LC-23 which showed distinc phyletic line with other type strain in Streptomyces genera.

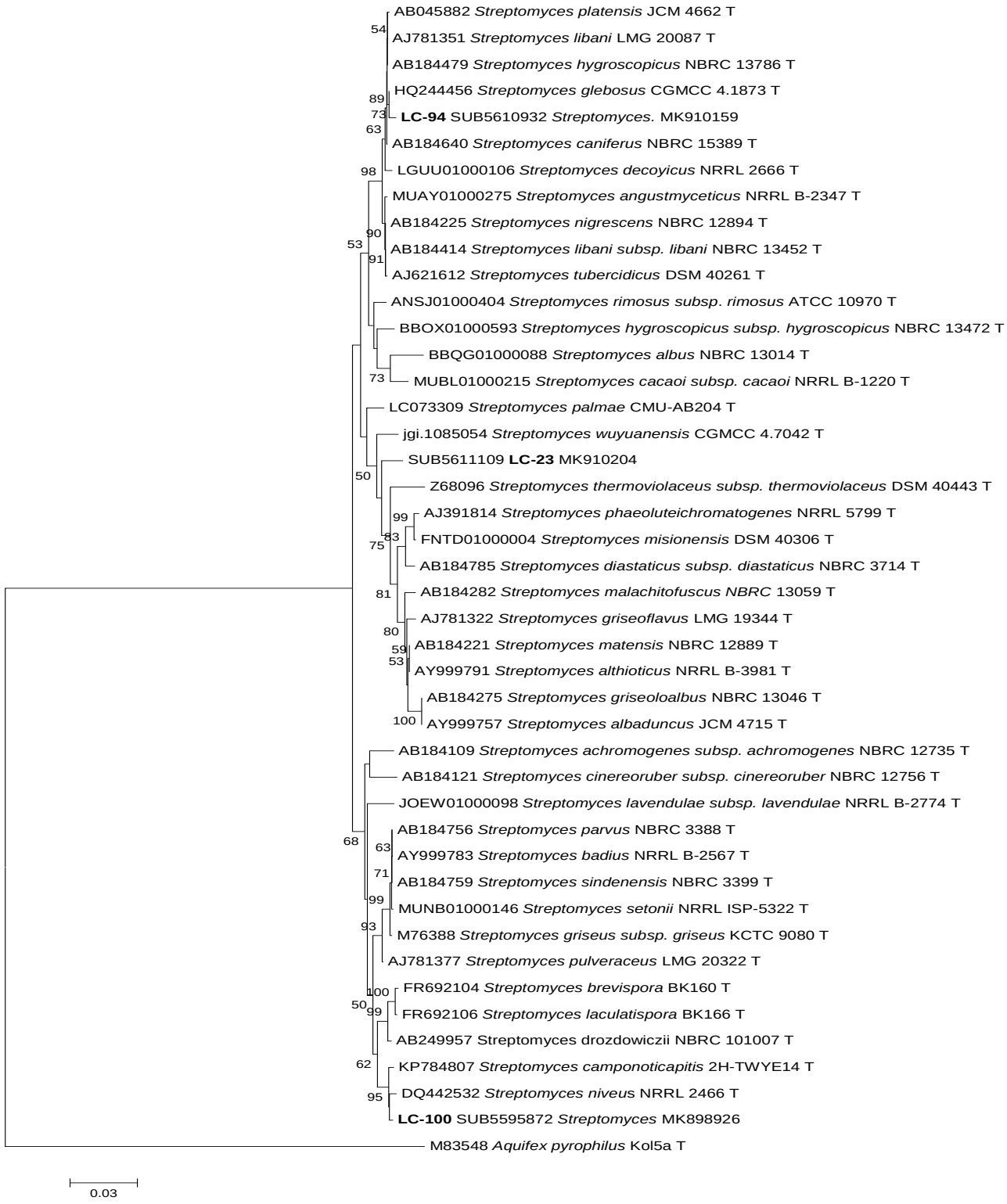

Figure 5. Phylogenetic tree constructed from 16S rRNA gene sequences of strain LC-23, LC-94, LC-100, and the type strain in the genus of *Streptomyces.*

DOI: http://dx.doi.org/10.14203/ann.bogor.2019.v22.n2.1-12

Discussion

Actinomycetes was succesfully isolated from tree lichen in area of Cibinong Science Centre (CSC) and Cibodas Botanical Garden (CBG), West Java, Indonesia. Spread dilution method on three kinds of agar media; HV, YIM 6 and YIM 711 obtained 125 isolates of lichens-associated actinomycetes. The combination of dilution number 10^{-3} until 10^{-5} and selective medium more affected in isolation process than specific thallus of lichen used in this research. Parrot *et al.,* (2015) isolated various actinomycetes from litoral lichens and declared that diversity of actinomycetes was most influenced by the selective media rather than lichen species or the level of lichen thallus association.

Seven genera of actinomycetes have been identified in this research; Actinoplanes, Amycolatopsis, Angustibacter, Kribbella, Micromonospora, Mycobacterium, and Streptomyces. Around 87% of the isolates belong to Streptomyces genera. Genus Streptomyces belongs to Streptomycetaceae family, in the classis of Actinobacteria and family Actinomycetales (Anderson and Wellington, 2001). Streptomyces species aerobic, most are able to torm extensively branched substrate mycelium and produce aerial hypae that typically differentiate into chains of spores (Kampfer *et al.,* 2008).

Antimicrobial activity was showed by 24 isolates against at least one microbial tested; to *Bacillus subtilis* BTCC B.612, *Escherichia coli* BTCC B.614, *Candida albicans* BTCC Y.33, *Staphylococcus aureus* BTCC B.611, *Micrococcus luteus* BTCC B.552. The potential isolates against more than one microbial tested has been shown by LC-23, LC-94, and LC-100 that belongs to Streptomyces genera. The most important characteristic of Streptomyces is the ability to produce secondary metabolite with antibacterial, antifungal, and antitumoral properties (Hasani *et al.,* 2014). Streptomyces produce 74% bioactive compound among another genera in Actinomycetes, and around 34% of all microbial metabolite (Berdy, 2005). Streptomyces species can be distinguished by many methods, such as molecular identification 16S rRNA (Hasani *et al.,* 2014). Molecular identification 16S rRNA of LC-23 has 98.51% similarity with *Streptomyces palmae*, LC-94 has 99.86% similarity to *Streptomyces caniferus* and LC-100 has 99.64% similarity to *Streptomyces camponoticapitis*. Some characteristic of those closests species were different with the characteristics owned by each isolates. Neighbour joining phylogenetic tree showed different result for LC-94. The closest species for isolate LC-94 was *Streptomyces glebosus* with 99.78% homology to LC-23. Interesting result of LC-23 showed distinct phyletic line with other Streptomyces species as type strain. All the closest species showed by phylogenetic tree have smaller similiraty value compared to 16S rRNA identification result.

The boundary for species delineation in genus Streptomyces seems to be higher than 97%. Identification 16S rRNA gene sequence data cannot serve as the sole basis for species delineation within the genus Streptomyces. Result of 'simple' treeing methods (the neighbour-joining method) should be regarded with caution, given that a tree is only a visual aid to place a novel species in its approximate neighbourhood (Kampfer and Labeda 2003). Berdy (2012) declared that only ~1% actinobacteria were cultivable. It was the reason why finding the novel species of Actinomycetes still an interesting research activity as the source of antibiotic producer. Indonesia lichens-associated actinomycetes was never been reported before. The result of this research may become a source to find potential bioactive metabolite as a new antibiotic.

Conclusion

Actinomycetes was sucesfully isolated from lichens in the area of Cibinong Science Centre (CSC) and Cibodas Botanical Garden (CBG). Totaly 69 isolates were identified as the genera *Actinoplanes, Amycolatopsis, Angustibacter, Kribbella, Micromonospora, Mycobacterium,* and *Streptomyces*. The screening process showed 24 isolates has antimicrobial activity, with the highest inhibitory activity against *Micrococcus luteus* BTCC B.552.

Acknowledgements

The author is grateful for the support to Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI) where the research conducted.

DOI: http://dx.doi.org/10.14203/ann.bogor.2019.v22.n2.1-12

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DOI: http://dx.doi.org/10.14203/ann.bogor.2019.v22.n2.1-12