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Characterization of *Micromonospora* spp. with Activity against Beta-Lactam Antibiotic-Resistant *Escherichia coli* ATCC 35218

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The emerge of antibiotic resistance has been an important issue all over the world, on the other hand, infectious diseases have been one of the highest causes of death causes in the world. Therefore, the discovery of a new antimicrobial drug is very important, and the group of rare actinomycetes are really promising as the producer of new bioactive compounds, in this case antibiotics. In this study we screened and characterized the actinomycetes with antibacterial activity against *Escherichia coli* ATCC 35218 resistant beta-lactam antibiotics. A total of 96 strains collected in Biotechnology Microbial Culture Collection (BioMCC), BPPT, were screened for their antibacterial activities by the agar plug method. Three strains, at-HH-64, at-HH-78, and at-HH-259, showed antibacterial activity. The selected strains were cultured on four different media, both solid and liquid media, e.g. ISP2, ISP4, Micromonospora Starch Medium (MS), and Bennet's Medium (BM), and we characterized their morphology and growth patterns. Morphological characterization showed that all strains belonged to the genera *Micromonospora*. The active strains were also identified based on 16S rRNA partial sequence. BLAST search of the 16S rRNA sequences of all tested strains with the sequences available in the NCBI data bank showed a maximum similarity 99% with *Micromonospora chersina*.

Key words : antibacterial activity, characterization, *Micromonospora,* screening

Munculnya resistensi antibiotik telah menjadi isu penting di seluruh dunia, di sisi lain, penyakit menular telah menjadi salah satu penyebab kematian tertinggi di dunia. Oleh karena itu, penemuan obat antimikroba baru adalah sangat penting, dan kelompok aktinomiset langka benar-benar menjanjikan sebagai penghasil senyawa bioaktif baru, dalam hal ini antibiotik. Dalam penelitian ini kami melakukan penapisan dan karakterisasi aktinomiset yang memiliki aktivitas antibakteri terhadap *E. coli* ATCC 35218 resisten antibiotik beta-laktam. Sebanyak 96 galur aktinomiset yang merupakan koleksi dari *Biotechnology Microbial Culture Collection* (BioMCC), BPPT, dilakukan penapisan aktivitas antibakteri terhadap *E. coli* ATCC 35218 dengan metode *agar plug.* Tiga galur menunjukkan aktivitas antibakteri, yaitu galur HH-64, HH-78, dan HH-259. Galur dengan aktivitas antibakteri selanjutnya ditumbuhkan di dalam empat jenis media, baik padat maupun cair, yaitu ISP2, ISP4, *Micromonospora Starch Medium* (MS), dan *Bennet's Medium* (BM), dan dilakukan karakterisasi morfologi dan pola pertumbuhannya. Karakterisasi morfologi menunjukkan bahwa seluruh strain termasuk ke dalam kelompok genus *Micromonospora*. Pada galur aktif juga dilakukan identifikasi berdasarkan sekuen 16S rRNA. Hasil BLAST sekuen 16S rRNA dibandingkan dengan data yang terdapat pada NCBI menunjukkan bahwa ketiga strain memiliki kedekatan tertinggi (99%) dengan *Micromonospora chersina*.

Kata kunci: aktivitas antibakteri, karakterisasi, *Micromonospora,* penapisan

Escherichia coli is a frequent cause of life-threatening bloodstream infections and other common infections, such as urinary tract infections. Antibiotic-resistant *E. coli* are readily acquired via the diet (food and water), and other factors such as continuous drug usage, the in appropriate dose usage, and the natural factors of that microbe itself. The resistance results in increased mortality, morbidity, and health care cost. In Indonesia resistant *E. coli* have emerged, whereas the rates of resistance to some antibiotics were significantly higher, especially to beta-lactam classes (Lestari *et al*. 2008). Beta-lactams remain the most widely utilized antibiotics owing to their comparatively high effectiveness, low cost, and minimal side effects. Among the various classes of antibiotics, penicillin, and cephalosporins are the most frequently used agents in treatment of bacterial infection. The most common mechanism of resistance to this class of antibiotics is the ability of bacteria to produce beta-lactamases, enzymes that degrade or modify the antibiotic before it can reach the appropriate target site. These enzymes are very important for Gram-negative bacteria, including *E. coli*, as they constitute a major defense mechanism against

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beta-lactam based drugs (Wilke *et al*. 2005). On the other hand the antibiotic resistant has been an important issue all over the world, on the other side, the discovery of new antibiotics is slower than the rate of resistance. Consequently, it is important to focus on screening programs of microorganisms, primarily of rare actinomycetes, for their production of antibiotics (Donadio *et al*. 2002).

Actinomycetes are well known as producers of bio-active secondary metabolites which include antibiotics, anticancer agents, enzymes, and biocontrol agents. According to Bérdy (2005), from 16 500 antimicrobial compounds isolated from microbes, 52.7% (8700 antimicrobial) were from actinomycetes, while the remaining 29.7 and 17.6% respectively were isolated from fungi and bacteria. Of 8 700 antimicrobial compounds isolated from actinomycetes, 6 550 have been isolated from the genus *Streptomyces* and the rest, 2 250 from rare actinomycetes. The discovery of a new antimicrobial compounds from rare actinomycetes have been reported by several researchers over the world (Hopmann *et al*. 2002; Beltrametti *et al*. 2006).

Micromonospora, which are a group of rare actinomycetes are widely distributed in nature, inhabiting variated environments such as coastal sediment (Zhao *et al*. 2004), soil rhizosphere (Qiu *et al*. 2008), peat swamp forest (Thawai *et al*. 2005), and from root nodules of the leguminous plants (Trujillo *et al*. 2007). *Micromonospora* species are best known for synthesizing antibiotics, especially aminoglycosides including gentamicin and netamicin, oligosaccharide antibiotics, and enediyne (Bérdy 2005).

In this study, we utilized the microbes collected in the Biotechnology Microbial Culture Collection (BioMCC), BPPT, specifically actinomycetes, as the source for screening of antibacterial compounds against *E.coli* ATCC 35218 resistant beta-lactam antibiotics, and we characterized the active strains.

MATERIALS AND METHODS

Actinomycetes Isolates. The strains were collection of Microbial Biotechnology Culture from the (BioMCC)-BPPT, isolated from soil using high-heating method, cultured on ISP2 agar medium, and maintained as frozen cell in 20% glycerol solution.

Screening of Antibacterial Activity. Antibacterial activity was assayed by the agar plug method on Mueller Hinton Agar (MHA). The ninety-six actinomycete isolates were subcultured on yeast-extract malt-extract agar medium (ISP2) for 14-21 d, and the agar containing the cells was cut off (about 7 mm in diameter) and placed on the assay plates inoculated with 10^6 CFU mL^{-1} tested bacteria. After 24 h incubation at 36 ± 1 °C, the antibacterial activities were determined by clearing zone around the tested isolates (Sharma *et al*. 2011).

Antibacterial Activity Patterns. The antibacterial activity pattern of *Micromonospora* spp. was conducted using the agar plug method (as described above). The active isolates were cultured on ISP2 agar medium and incubated at 28 °C for 15 d. Every 3 d of interval incubation, the plates were picked up and assayed their antibacterial activities againts test bacteria. For the antibacterial activities were expressed as the diameter of the growth inhibition zone (mm) and measured using calipers (Mitutoyo, Japan).

Morphological and Growth Pattern Characterization of Active Actinomycetes. The potent actinomycetes isolates were selected and characterized by morphological characteristics in four different agar media (yeast-extract malt-extract (ISP2), inorganic salt-starch (ISP4), *Micromonospora* Starch Medium (MS), and Bennett's Medium (BM)). The morphological methods consist of macroscopic and microscopic characterization. Macroscopically, the actinomycete isolates were grown in four different media, and characterized their growth and colony color after 21 d of incubation. For microscopy, the plates from different media were observed for their mycelial structure, spores, and sporophore development directly under a microscope (Olympus BX51, 1000× magnifications). The observed morphology of the isolates was compared with the Actinomycetes morphology provided in Bergey's Manual for the presumptive identification of the isolates (Kawamoto 1989). For growth patterns characterization, a pure culture of the actinomycetes isolates was inoculated individually into the seed medium (ISP2 broth). After 72 h of incubation, the seed culture at a rate of 10 % (v/v) was transferred into the four different fermentation media, using the same media as for morphological characterization (described before). The fermentation was carried out at 28 °C for 15 d under agitation at 220 rpm (performed using Rotary Shaker Incubator, Takasaki Scientific Instruments Corp). After every 3 d of incubation, the flasks were harvested, and the amount of packed mycelial cells (PMV) was measured. Culture broth was taken (10 mL) and centrifuged at 3 000 x g for 5 min (performed using Kubota 7780) to separate cells from fine particles of the medium. The separated mycelia were resuspended in a saline solution (0.85 % sodium

chloride) to give the original volumes using 15 mL centrifuge tubes. This step were replicated three times, then the mycelia was allowed to sediment for 20 min. The PMV was measured and expressed as % of cells to total volume (10 mL) (Rho *et al*. 1991).

Molecular Identification of Active Strains Based on 16S rRNA Sequence. The DNA from active actinomycetes was isolated using InstaGene Matrix (BioRad). The pure colony of the isolate was collected from fresh culture on ISP2 agar medium, transfered to the microtubes consisting of 10 µL ddH_2O, added with 90 µL Instagene Matrix, and heated at 100 °C for 15 min. The mixed solution was centrifugated at 15 000 x g (TOMY MX-301) for 3 min. The DNA amplification was performed via Polymerase Chain Reaction (PCR) by PCR Thermal Cycler (Takara, Japan). The DNA template (8 µL) was added to the amplification mixture containing 1.6 µL ddH_2O, 1 µL DMSO, 0.2 µL primer 178 F, 0.2 µL primer 179 R, and 9 µL Master Mix. After the initial denaturation for 2 min at 96 °C, 35 cycles of amplification were performed, 20 s at 90 °C denaturation, 20 s at 60 °C annealing and 1 min 72 °C elongation. These cycles were followed by a final 5 min elongation cycle at 72 °C. The PCR product purification was done using Gel/PCR DNA fragments Extraction Kit (DF Buffer, Washing Buffer, Elution Buffer, DF Column) and amplified by PCR using the mixture reaction from Go Taq Green Master Mix (Promega). The PCR product was directly sequenced using ABI 3130 Genetic Analyzer, and 16S rRNA sequences data from each isolate was compared to the database available at GenBank by using the BLAST software (blastn) on the National Center Biotechnology Information site (NCBI) (http://www.ncbi.nlm.nih.gov/). The 16S rRNA sequences of the active strain were aligned using the CLUSTAL W2 program. Phylogenetic and molecular evolutionary analyses were conducted using MEGA software version 5. The evolutionary distances were computed using the Maximum Composite Likelihood method and were in units of the number of base substitutions per site (Tamura *et al.* 2011).

RESULTS

Antibacterial Activity of *Micromonospora* spp. From the ninety-six actinomycetes strains which were assayed, three strains showed inhibition activity against *E.coli* ATCC 35218, they were strains HH-64, HH-78, and HH-259, with the inhibition diameters of 13.55, 13.88, and 14.71 mm, respectively (Fig 1). Characterization of antibacterial activity, based on the age of culture (Fig 2) showed that the highest activities were at 9 d of culture. Antibacterial activities started on the third day of culture, except for the strain HH-259, and continued to increase until the 9 d of culture. The antibacterial activities began to decline at 12 d of culture. Overall, up to 15 d of culture, all of the strains still showed their antibacterial activities.

Morphological and Growth Patterns Characteristic of *Micromonospora* spp. *Micromonospora* spp**.** showed varied growth patterns in different liquid media, indicated by the yielded biomass (% PMV). The strain HH-64 showed the best growth in ISP4 broth, followed by the growth in the ISP2, MS, and BM media. This pattern also occured for strain HH-259. The best growth medium for strain HH-78 was ISP4, followed by ISP2, BM, and MS (Fig 3). Macroscopically, morphological colony observations of three strains in the four different growth media varied. Strain HH-64 grew abundantly in all media and sporulated more slowly on Bennet's medium. Strain HH-78 grew abundantly and sporulated well in all media. Strain HH-259 grew abundantly on ISP2 medium and moderately on three other media, and it sporulated slowly on MS and BM media. Overall, the color of vegetative mycelium of the strains was colorless to light orange-

Fig 1 The antibacterial activities of *Micromonospora* spp. against *E. coli* ATCC 35218 (shown by clear zones around the tested strains).

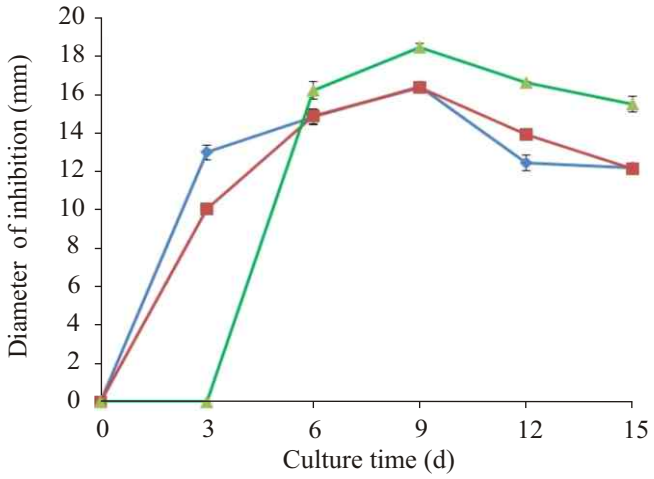

Fig 2 Antibacterial activity patterns of *Micromonospora* spp. from ISP2 agar culture performed using agar plug method. —◆—: HH-64; —■—: HH-78; —▲—: HH-259.

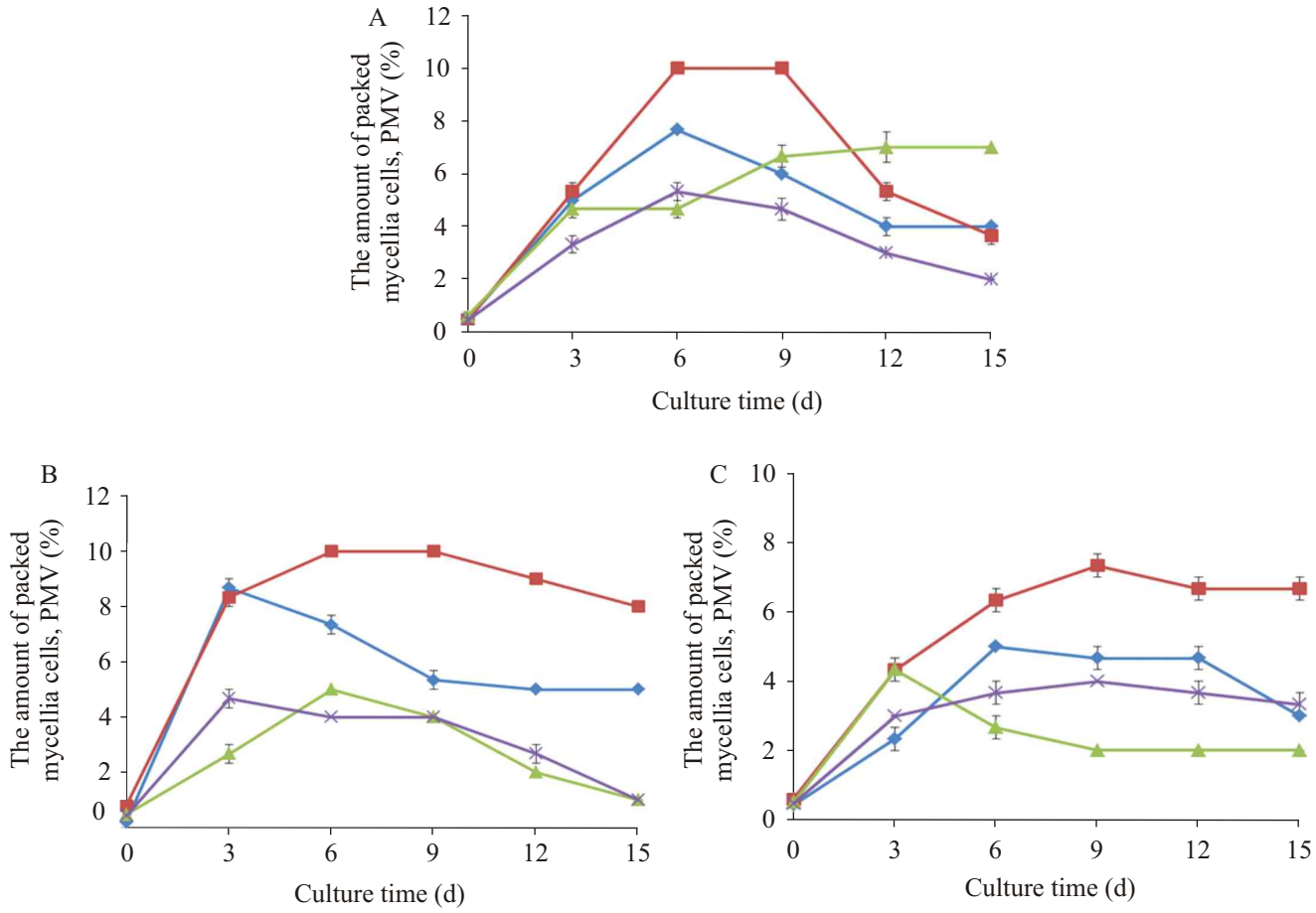

Fig 3 The growth patterns of *Micromonospora* spp. in four various liquid media (ISP2, ISP4, Micromonospora Medium (MS), and Bennet's Medium (BM). (A) Isolate HH-64; (B) Isolate HH-259; (C) Isolate HH78. —◆—: ISP2; —■—: ISP4; —▲—: MS; —✳—: BM.

yellow, then turned to greenish-black or brownish-black after sporulation (Fig 4). Microscopic observation showed that all active strains belong to the genus *Micromonospora* (Fig 5).

Molecular Identification of *Micromonospora* spp. The partial sequences of each strain was compared with 16S rRNA gene sequences in the GenBank database. The homology search by the BLAST program showed that all strains showed similarities to *Micromonospora chersina,* with 16S rRNA gene-sequence-similarity values of 99%. The results indicated that the closest related species for strain HH-

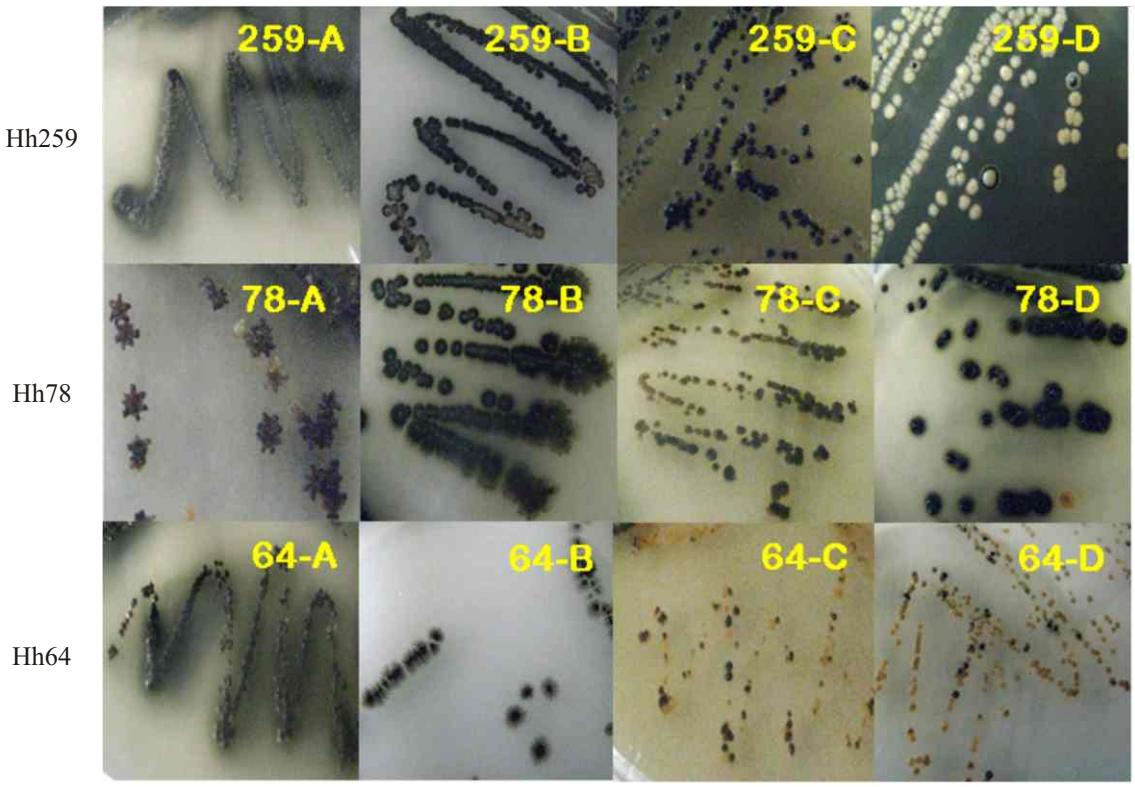

Fig 4 *Micromonospora* spp. colonies after 21 d incubation on four different agar media (A. ISP2; B. ISP4; C. Micromospora Medium; D. Bennet's Medium).

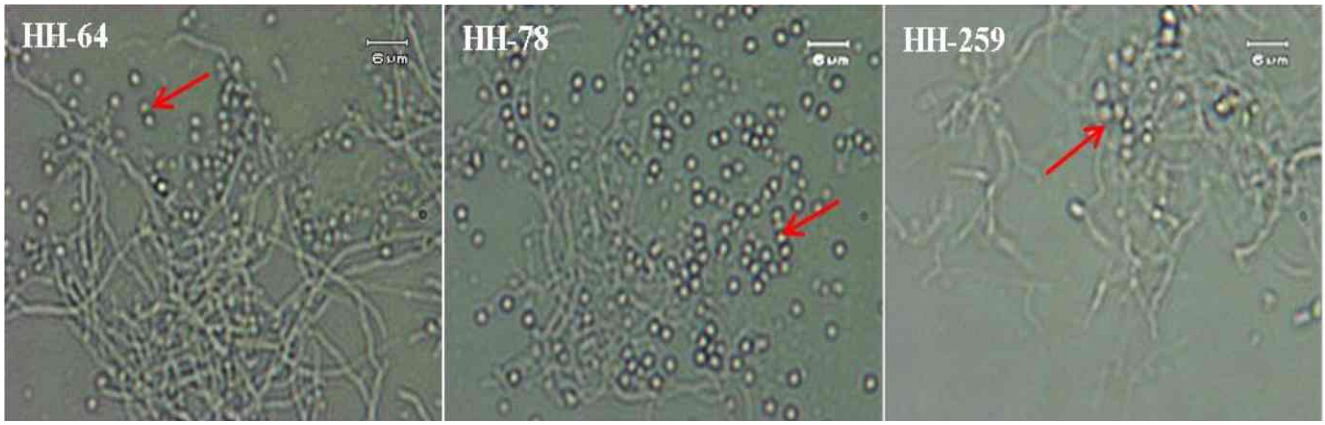

Fig 5 Microscopic structures of *Micromonospora* spp. 21 d old on ISP2 medium, the spherical spore indicated by the red arrow (1000 x magnification).

64 was with *M. chersina* R-Ac138 (Fn649453), for strain HH-78 with *M. chersina* RtIII8 (EU274367), and for strain HH-259 was with *M. chersina* R-Ac135 (Fn649452), respectively. The phylogenetic dendogram was inferred using the Neighbor-Joining method and the results showed that two strains, HH-64 and HH-259, were clustered together and were closely related with other *M. chersina.* The strain HH-78 was separated from the anothers and it was clustered together with *M. olivasterospora, M. viridifaciens,* and *M. echinaurantiaca* (Fig 6).

DISCUSSION

From the results we obtained, 3 strains showed antibacterial activities, and the patterns of antibacterial activity based on the age of the cultures showed that until 15 d of culture they still have activities against the target bacteria. Antibacterial activities are characterized by the formation of a clear zone around the actinomycetes colony being tested (Fig 1), the wider the clear zone formed, the greater the antibacterial activity produced. This indicates that the strains are capable of secreting a

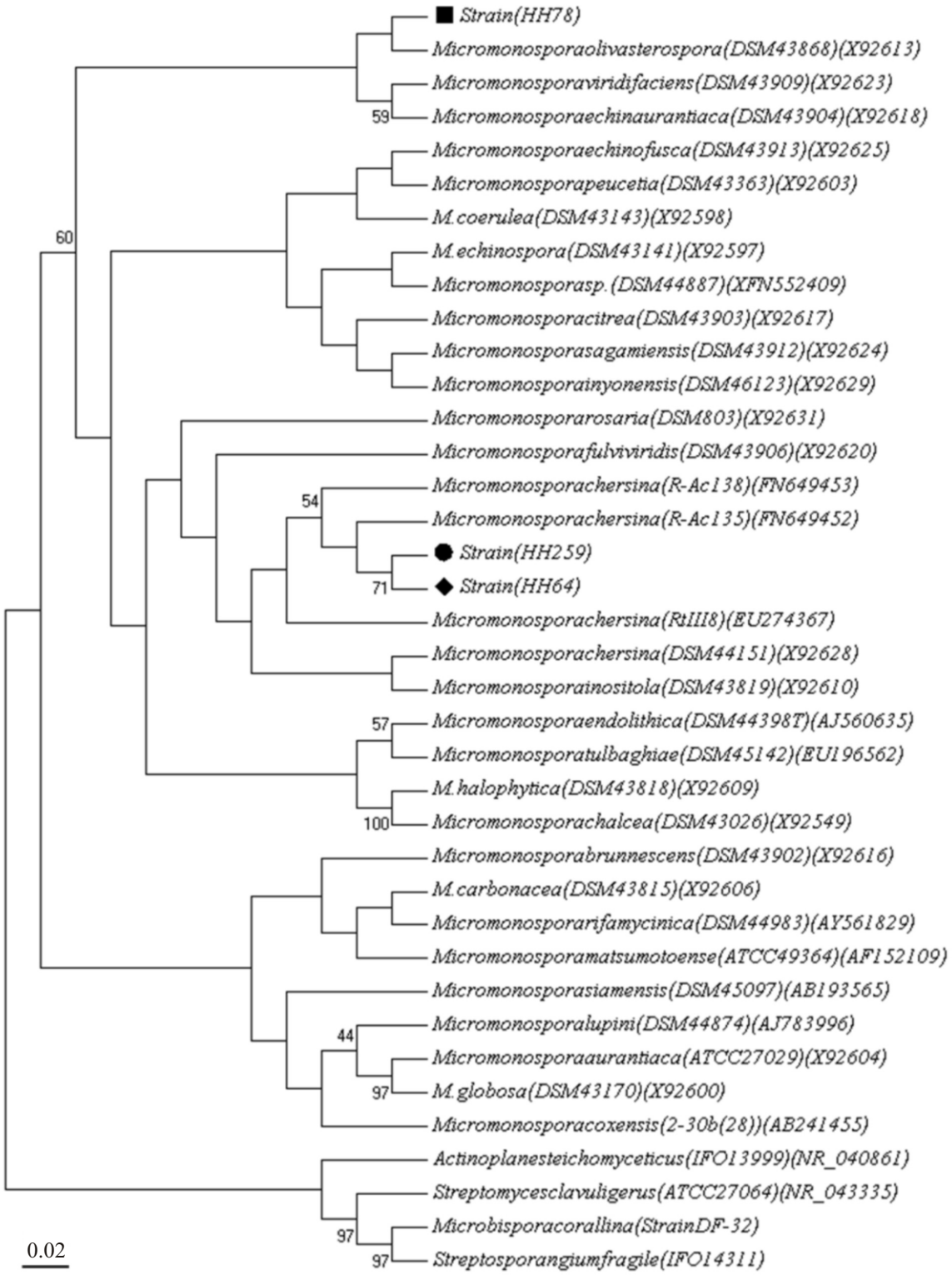

Fig 6 The phylogenetic dendogram based on 16S rRNA gene sequences of *Micromonospora* spp. and other genera (root of bootstrap). The strains are shown in bold symbols. Percentage bootstrap values based on 500 resampled data sets are shown at the nodes; bootstrap value lower than 40% were cut off.

substance that inhibits the growth of test bacteria, in this case *E. coli* ATCC 35218. The bioactive compounds could be a beta-lactamase inhibitor or antibacterial compounds that cannot be hydrolyzed or are hydrolysed poorly by beta-lactamase activity produced by the target bacteria (Babic *et al*. 2006).

The bacterial target we used in this study was *E. coli* ATCC 35218, it is a beta-lactam resistant bacteria, a non-pathogenic that produces TEM-1 beta-lactamase. This bacteria is used as a bacterial surrogate for EHEC *E. coli* O157:H7 in microbial challenge studies (Gurtler *et al*. 2010). In Gram-negative

bacteria, beta-lactamases are the most important mechanism of resistance to beta-lactam antibiotics. Beta-lactamases are bacterial enzymes that hydrolyse the beta-lactam ring and render the antibiotic inactive before it reaches the penicillin-binding-protein (PBP) target. There are two principal ways to overcome the beta-lactam resistance involving finding inhibitors or inactivators of beta-lactamases and finding a new beta-lactam antibiotic that demonstrates great affinity for the target PBP which is not hydrolized or hydrolized poorly, by beta-lactamases (Babic *et al*. 2006). Currently, there are 3 beta-lactamasei inhibitors used clinically: clavulanic acid, sulbactam, and tazobactam, which are combined with other antibiotics (Miller *et al*.2001).

Antibacterial agents are one of the secondary metabolites produced by actinomycetes. Secondary metabolites are produced as a response to the limited availability of nutrients, and are not essential for the growth of the producing cultures but serve diverse survival functions in nature. In contrast with secondary metabolites, the primary metabolites are associated with growth and maintenance of life, its concern with the release energy and the synthesis of important macromolecules such as sugars, protein, nucleic acids, and organic acids. The organism will be die if primary metabolism were stopped (Demain and Fang 1995). In actinomycetes, the production of secondary metabolites coincides with, or precedes, sporulation. Its occurs when cultured on the agar medium. In the liquid culture, the secondary metabolites generally limited to the stationary phase, and its frequently assumed to the result of nutrient limitation. Because both secondary metabolites production and sporulation occurs close to the beginning of the stationary phase, one may suspect that these two process are regulated by an overlapping mechanism. However, in some cases there is no close connection between the spore formation and antibiotic production, particulary, by non-sporulating organisms (Bibb 2005; Glazer and Nikaido 2007).

The growth pattern of active strains showed that the patterns varied in different media (Fig 4). The best growth is demonstrated by the growth in ISP4 medium. The medium has a more complex composition than other media we used, it contains soluble starch, ammonium sulphate, and potassium phosphate, as a source of carbon, nitrogen, and phosphate, respectively. In addition, the media also provides micro-elements as a sources of metals, such as ferrous iron and manganese. The growth patterns in this medium also varied among the strains, which may due to their ability to assimilate the medium components variably. Microbial growth and antibiotic production by actinomycetes and other spore forming strains in batch culture are mainly influenced by two factors. The first is the nature and concentrations of different medium composition such as carbon, nitrogen, phosphate, and metals sources. Carbon sources that can readily serve as growth substrates, such as glucose, often repress antibiotic production but are excellent for growth. Polysaccharides such as, starch, are often preferable in antibiotic yield. The second factor is cultivation conditions such as temperature, pH, incubation time, oxygen supply, type and concentration of inoculum. In shaking flask cultures, the important factors that influence the gas-mass transfer in the cultivation media are mainly the shape and volume of the cultivation vessel, medium volume, the degree of agitation, and the medium viscosity (El-Enshasy *et al*. 2000; Himabindu and Jetty 2006).

In order to maximize the fermentative production of secondary metabolites, in this case antibacterial compounds, it's important to characterize the producing organism. Characterization of the active strains showed that all strains belonged to the genus *Micromonospora* and they closely related to *M. chersina.* Microscopically, they were characterized by the presence of single spherical spores located terminally on short or long hyphal branches and sometimes developed into clusters,with no aerial mycelium being formed. *M. chersina* was first proposed by Tomita *et al*. (1992), the vegetative mycelium of this strain is branched monopodially and forms single spores (spherical with short blunt spines) which are sessile or born on short- or long-monopodial sphorophores, and no aerial mycelium is formed. This strain is known as a dynemicin producer, an antitumor antibiotic, with broadspectrum activity. Not only did it have activity against tumor cells, it also had activity against bacteria and fungi (Konishi *et al*. 1991). The phylogenetic analysis (Fig 6), showed that the strain HH-78 was separated from the two others and its closely related species, *M.chersina*. Instead, it clustered together with *M. olivasterospora, M. viridifaciens,* and *M. echinaurantiaca,* this was probably due to their menaquinone concentration and fatty acid composition (Tomita *et al*. 1992; Koch *et al*.1996).

Besides *Streptomyces*, *Micromonospora* is one of the genera of actinomycetes that is known as world antibiotics producer and this genus has remained poorly studied compared to *Streptomyces.* Some new

bioactive compounds have been isolated from *Micromonospora* species, and have been shown to have antitumor, antiparasitic, and antimicrobe activity (Charan *et al*. 2004; Igarashi *et al*. 2007; Gärtner *et al*. 2011; Sakai *et al*. 2012). Other members of the genus *Micromonospora* that known as antibiotic producers were *M. purpurea* and *M. echinospora*, as gentamicin producers, and *M. chalcea* as a izumenolide producer. Gentamicin is a broad spectrum, basic, and water soluble antibiotic, which highly activity against Gram negative bacteria and *Mycobacterium tuberculosis* (Himabindu and Jetty 2006), meanwhile, izumenolide is beta-lactamase inhibitor (Liu *et al*. 1980).

In this study we have shown that rare actinomycetes, specially, *Micromonospora* from Indonesian habitat are potential antibacterial producers, in this case, as *E.coli* resistant beta-lactam antibiotics. Further studies are needed to obtain the optimum media and conditions of fermentation production, isolation, purification, and characterization of the active compounds.

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