BIODIVERSITAS
Volume 22, Number 9, September 2021
Pages: 3785-3791

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d220923

A salt tolerant *Sphingosinicella microcystinivorans* A3 isolated from soil contaminated with mercury in traditional gold mining of Jendi Village, Wonogiri District, Indonesia

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Manuscript received: 21 July 2021. Revision accepted: 21 August 2021.

Abstract. *Sutami, Purwanto, Rosariastuti R. 2021. A salt tolerant Sphingosinicella microcystinivorans A3 isolated from soil contaminated with mercury in traditional gold mining of Jendi Village, Wonogiri District, Indonesia. Biodiversitas 22: 3785-3791.* Isolation and characterization of indigenous bacteria from the soil of traditional gold mining contaminated with mercury is the first step in a series of research to explore and utilize indigenous bacteria in Jendi's area. This study was aimed to determine the characteristics and identity of bacterial isolates from soil of traditional gold mining in Jendi Village, Wonogiri contaminated by mercury. The methods used in this study included bacterial isolation, media preparation, phenotypic identification including; morphological and physiological tests and genotyping tests. The results showed that the bacterial isolate A3 grew optimally in media with the addition of 10% NaCl, at a temperature of 27°C, and pH 9. There were negative reactions to the observations of gram staining, acid production from glucose, indole production, catalase and urease, and positive reactions to oxidation. A neighbor-joining phylogenetic tree based on the 16S rRNA gene sequence showed that the A3 strain was closely related to *Sphingosinicella microcystinivorans* strain $Y2^T$ ($JCM\ 13185^T$) with 100% Query coverage and a maximum identity of 99.56%.

Keywords: Gold-mining, mercury, PCR, salt tolerant, *Sphingosinicella*

INTRODUCTION

Indonesia is a country rich in natural resources, including mining materials (Ma'arif 2014) and is known as the largest coal and gold producer in the world (Dutu 2015). The mining industry in addition to provide many benefits, also causes environmental damage (Khanifah et al. 2020). Heavy metal is a type of pollutant widely distributed in the soil and gets special attention because of its non-degradable nature and can lasts for a long time in the environment (Wang et al. 2020). Different heavy metals including Cr, Cd, Pb, Hg, and As are often reported to contaminate the environmental habitats (Li et al. 2020). Mercury (Hg) is among the one of the most toxic pollutants (Selid et al. 2009; Tchounwou et al. 2012; Waheed et al. 2020). That is widely distributed in the atmosphere, lithosphere, and surface water. Mercury causes serious problems for human health, such as bioaccumulation in the brain and kidneys which ultimately leads to neurological diseases (Zulaikhah et al. 2020).

Gold mining in Jendi (Village) of Selogiri Sub-district and Wonogiri District is operating without any permit (Dewi et al. 2013). Gold mining activities are processed using an amalgamation process. The amalgamation process can cause negative impacts in the form of environmental pollution by mercury vapors. As much as 10 - 30% of the mercury used in the gold amalgamation process released to the environment (Alpers et al. 2005). The amalgamated water is dumped into ditches and flows into rivers, which are used to irrigate the surrounding paddy fields, as a result, this area has been contaminated by mercury. As per reports obtained from analyses of thirty soil samples in Jendi Village revealed that the soil contained Mercury with an average of 30.87 $mgkg^{-1}$. Based on Government Regulation No. 101 of 2014 Concerning Management of Hazardous and Toxic Waste, the threshold for mercury levels in the soil is 0.3 $mgkg^{-1}$ (Rhani 2012). Mercury levels in river water around Jendi Village vary from 0.0024 mgL^{-1} to 0.0173 mgL^{-1}. Government Regulation No. 82 of 2001 concerning Water Quality Management and Water Pollution Control, the mercury threshold in water is 0.001 mgL^{-1}. Thus, mercury levels in the soil and river water in Jendi Village have exceeded the predetermined threshold. Therefore, bioremediation is an effective and safe alternate to deal with this problem. The efforts can be carried out by isolating indigenous bacteria from the local environment which is expected to help in overcoming pollution problems that occur in the region (Winardi et al. 2020). This study aims to determine the characteristics and identity of bacterial isolates obtained from mercury-contaminated traditional gold mining in Jendi Village, Wonogiri.

MATERIALS AND METHODS

Sample collection

Samples were collected during April 2020 from mercury-contaminated traditional gold mining in the

village of Jendi, Selogiri, Wonogiri, Indonesia (07°47'41.9"S, 110°52'51.1"E). Soil and wastewater samples were taken randomly in several areas where mining materials were processed. Based on the results of the analysis, the soil and wastewater contained mercury of 10.98 $mgkg^{-1}$ and 0.65 mgL^{-1}. Soil samples were taken from the rhizospheric soil of plant grows well in sampling location with a depth of 20 cm using a soil drill. Soil and wastewater samples were put into sterile containers and stored in a cool box. This is done to reduce the presence of bacterial contamination from the air.

Instruments and culture media used and sterilization

The tools used in this research were UV-Vis Spectrophotometer (Cory Conc 50), incubator (Heraeus), Autoclave (Astell), oven (Memmert), laminar airflow (Esco), analytical balance (Kern), microscope, pH digital meters, refrigerators, micropipettes, 100°C thermometers, Petri dishes, Bunsen, horn spoons, loop needles, spray bottles, sterile sample bottles and glassware commonly used in laboratories. Two different liquid media were used: A Nutrient Agar (NA); and a soil extract medium (SEM). NA was prepared as described previously by Fan et al. (2002) and SEM was prepared using soil and wastewater from the field site in gold mining in Jendi, Wonogiri. Antifungal, 70% alcohol, sterile distilled water, Kovac's reagent, methyl red reagent, and yeast extract, SIM Medium, Tryptone broth, Christensen's Urea Agar, Stuart's Urea Broth. All glassware used were wrapped in paper, and then sterilized using an autoclave at 121ºC with a pressure of 1.5 bars for 30 minutes. Medium Nutrient Agar (NA) was measured as needed, then heated until dissolved. The medium was sterilized by autoclaving at 121ºC at 1.5 bars pressure for 15 minutes.

Soil bacterial isolation

The isolation of soil bacteria was carried out using soil extract agar (Hii et al. 2020). Soil samples that had been taken from 5 points at each sampling location were homogenized, and then 5 g were taken and dissolved in 45 ml of physiological NaCl solution, then vortexed (10^{-1} dilution). The 10^{-1} soil suspension results were made in graded dilutions up to 10^{-11} using physiological NaCl solution. The results of 10^{-3} to 10^{-11} dilutions were then cultured on Nutrient Agar (NA) media, then incubated at room temperature for 24 hours. Bacterial growth was carried out using the pour plate technique. Colonies that grew in the range of 25-250 colonies were then observed for bacterial morphology and counted the number of bacterial colonies to determine the number of bacterial isolates obtained. Bacteria obtained from mixed cultures were separated using the streak quadrant method using a blunt loop and the media used was NA media. Separated colonies on scratch plates were transferred to slanted NA media which was used as stock culture (Aanniz et al. 2015).

The bacterial colonies that grew separately were then tested for their resistance to growth in NA media containing 10 $mgkg^{-1}$ Mercury. The bacteria that managed to grow were purified to obtain pure isolates. Purification of isolates using streak plate method on NA medium. Pure isolates were then identified based on characteristics that referred to Bergey's Manual of Determinative of Microorganisms (Ludwig et al. 2010).

Bacterial identification

The identification and characterization of the selected isolates was carried out by both phenotypic test and the genotypic test.

Phenotypic characterization

The phenotypic characterization tested in this study included macroscopic observations including colony morphology, microscopic observations including cell shape and cell staining, after macroscopic observations, further tests were carried out only for isolates that grew best in media containing mercury. Further tests in the form of physiological tests include; oxygen demand test, carbohydrate fermentation ability, NaCl resistance test, and biochemical tests; motility test (Public Health England 2014), catalase test (Reiner 2010), oxidase test (Shields and Cathcart 2016), testing the ability to hydrolyze urea (Brink 2010), indole test (MacWilliams 2009), and acid production from glucose (Public Health England 2019).

Genotypic characterization

Genotype characterization was done by 16S rDNA gene sequencing. Genomic DNA of bacterial isolates was extracted from 5 mL bacterial cultures grown overnight following the previously described method. Bacterial pellets were suspended in 410 µL TE buffer (10 mM Tris-HCl pH 8 and 1 mM EDTA pH 8). A volume (50 µL) of 60 $mg.mL^{-1}$ fresh lysozyme (Sigma, Milwaukee, WI) solution was added to cells suspension and incubated. for 30 minutes at 37°C with occasional mixing. Subsequently,30 µL of 10% SDS (Sodium Dodecyl Sulfate) and 3 µL of 20 $mg.mL^{-1}$proteinase K were added, mixed and incubation was continued for 30 min. After that, 100 µL of NaCl 5 M and 100 µL of CTAB (N-cetyl-N, N, N, -trimethylammonium bromide) 10% were added, slowly mixed, and incubated at 65°C for 10 minutes. The mixture was extracted with 600 µL of ice-cold chloroform by gently mixed, centrifuged at 12,000 rpm for 5 min, and the aqueous phase was carefully removed and transferred to a new sterile micro centrifuge tube. The DNA in the aqueous phase was precipitated by adding 0.6 vol. ice-cold isopropanol and incubating for one hour on ice. The precipitate washed with ice-cold 70% ethanol. After drying, the precipitate was resuspended in 50 µL of TE buffer.

Universal bacterial 16S rRNA primers, 27 F (AGAGTTTGATCMTGGCTCAG) and 1492 R (TACGGYTACCTTGTTACGACTT) used to manage implication fragment of the 16S rRNA gene in genomic DNA of bacteria were isolated as a template to follow proto cabbage (Stackebrandt and Goebel 1994) with modifications in men NOTICE 2x My Taq HS Red Mix (Bioline, BIO-25048). PCR was performed in the GeneAmp@PCR system 9700 (Applied Biosystems, Foster City, CA). The following conditions were used for DNA

amplification. Initial denaturation at 95°C for one minute, followed by 35 cycles consisting of 15 seconds at 95°C, 30 seconds at 52°C, plus an additional final extension step of 45 seconds at 72°C. The accuracy of the PCR product (approximately 1400 bp) was confirmed by electrophoresis through a 0.8% horizontal agarose gel containing 0.5 g.mL-1 ethidium bromide. The gel was examined under UV light and photographed. The PCR products were purified using Zymoclean ä Gel DNA Recovery Kit (Zymo Research).

Sequencing of 16S rRNA was performed on the ABI PRISM 3100-Avant™ Genetic Analyzer (Applied Biosystems, Foster, CA) with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Primer used for sequencing of 16S rDNA was 27 F (AGAGTTTGATCMTGGCTCAG) and 1492 R (TACGGYTACCTTGTTACGACTT). Several alignments of the order (ca. 715 basis) were determined performed with the program GENETYX WIN Ver. 3 (Software Development, Tokyo, Japan). A GenBank search with the BLAST program (www.n cbi.nih.nlm.gov/Blast.cgi) was used to identify the named bacterial species. X2.0 clusters (ftp://ftp.ebi.ac.uk/pub/software/clusterw2) were used to construct a phylogenetic tree and then drawn using a neighbor-joining NJ Plot (http://pbil.univ lyon1.fr/software/njplot.html)

RESULTS AND DISCUSSION

Phenotypic characterization

Morphological observation

The results of morphological observations of 3 bacteria isolated coded T (*Cassava* rhizosphere) and A (wastewater) are shown in Table 1.

After the isolates were incubated for 24 hours, 3 types of isolates were obtained that could grow on media that had been given Mercury, namely T5, T7, and A3. One of the three bacterial isolates named as A3 grew well in media containing Mercury. Colonies of the isolates T5 and T7 are taken from the soil rhizosphere of cassava plants showed characteristics features as T5 has the round shape, elevation flat, shiny surface, flat edge and creamy white, while, the T7 has a round shape, elevation as droplets, shiny surfaces edge smooth and creamy white.

A3 isolate was an isolate taken from traditional gold mining wastewater and had a round shape, convex elevation, glossy surface, flat edge, and yellow color. These characteristics are close to those of the genus *Sphingosinicella*. Members of the genus *Sphingosinicella* are Gram-negative, rod-shaped, and highly aerobic. *Sphingosinicella humi*. has white, smooth, circular, convex, and slightly transparent colonies on R2A media (Qiao et al. 2007). *Sphingosinicella xenopeptidilytica* has pale yellow colonies, round and convex. Cells are aerobic and measure 0.6-0.861, 5-2.5 mm (Geueke et al. 2007). *Sphingosinicella gene* has yellow colonies, truly aerobic and chemo-organ trophic (Maruyama et al. 2006).

Microscopic observations

Microscopic observations were made by Gram staining. The results of microscopic observations of bacterial isolates obtained are shown in Table 2.

Bacteria are grouped into two groups, namely gram-positive bacteria and gram-negative bacteria. Gram staining is based on differences in the structure of the bacterial cell wall, resulting in different reactions in the permeability of the dye and the addition of a washing solution. Gram-positive bacteria's cell wall consists of a thick layer of peptidoglycan while the cell wall of Gram-negative bacteria has thick lipid content.

The results of microscopic observations (Table 2) showed that, all bacterial isolates belong to the Gram-negative group of bacteria. In addition, bacterial isolates formed single colonies with the shape of rod cells (*Bacillus*) in isolate A3 while isolates T5 and T7 were spherical (*coccus*). To give optimal results in Gram staining, it is better to use fresh cultures aged 24-48 hours, because in old cultures many cells are damaged in their cell walls, this results in the release of dye when washed with a bleach solution, thus disguising the results. According to Geueke et al. (2007), *Sphingosinicella xenopeptidilytica* is Gram-negative bacteria, as determined by KOH and aminopeptidase assays. According to Maruyama et al. (2006) *Sphingosinicella* gene has rod cell shape, Gram-negative, does not form spores, motile using polar flagella and completely aerobic and chemo-organ trophic.

The discovery of Gram-negative isolates in Jendi water sources related to environmental conditions. Gram-negative bacteria require relatively simple nutrition compared to Gram-positive bacteria. This means that the ability of this group of bacteria to grow in an environment is greater than that of Gram-positive bacteria.

Biochemical test

The biochemical tests were carried out for the confirmation and identification of the bacteria isolated. The results of the observation of the biochemical test of bacterial isolates can be seen in Table 3.

Table 1. Results of observation of bacterial colony morphology

Isolate Code	Colony form	Elevation	Surface	Edge	Color
T5	Round	Flat	Glossy	Flat	Creamy White
T7	Round	As droplets	Glossy	Flat	Creamy White
A3	Round	Convex	Glossy	Flat	Yellow

Table 2. Results of microscopic observations of bacterial isolates

Isolate code	Cell shape	Gram's properties
T5	*Coccus* (round)	Gram-negative
T7	*Coccus* (round)	Gram-negative
A3	Basil (stem)	Gram-negative

Table 3. Biochemical Test Results of A3 Bacterial Isolates

Observation Parameter	Result
Catalase Test	+
Oxidase Test	+
Urea hydrolyzing Test	-
SIM test:	
Sulfur reduction	-
Indole test	-
Motil test	+
Acid production from glucose	-

Note: (+): indicates positive, (-): indicates negative

Catalase test

The catalase test was carried out to obtain a more specific information of genus of the bacterial isolates. Based on the results of the study, bacterial isolates showed positive results, indicated by the formation of many oxygen bubbles shortly after the addition of 3% H_2O_2 solution (Reiner 2010). This means that bacterial isolates can produce catalase enzymes and have tolerance for oxygen availability. Catalase enzyme is an enzyme that can catalyze the direct conversion of hydrogen peroxide (H_2O_2) which is toxic to cells into water and oxygen (Lionel 2016). According to (Qiao et al. 2007) *Sphingosinicella microcystinivorans* JCM 13185T; *S. soli* KSL-125T, *Sphingomonas paucimobilis* JCM 7516T had a positive catalase test.

Oxidase test

An oxidation test was carried out for the Gram-negative bacterial isolate. Based on the results, bacterial isolates showed positive results, which were indicated by the appearance of a purple color on the surface of the filter paper (Shields and Cathcart 2016). This means that the isolate belongs to a group of bacteria that have cytochrome oxidase enzymes. Cytochrome oxidase is a complex enzyme that plays a role in oxidative phosphorylation. According to Mayugama (2006), the bacteria that produce cytochrome oxidase, namely *sphingosonicella.* Oxidase-positive bacteria allow them to oxidize reagents containing certain amine

Urea hydrolyzing test

The urea test on bacterial isolates gave a negative result in the form of the color of the media that did not change, namely yellow; this was because the bacterial isolates did not produce the urease enzyme. Microorganisms that produce the enzyme urease will break down urea into ammonium and CO_2. When urea is hydrolyzed, the culture medium containing urea and a pH indicator (phenol red) accumulate ammonium in the culture medium and cause the pH to become alkaline. The color change from yellow to pink is an indication of urea hydrolysis (Brink 2010). According to Qiao et al. (2019), *Sphingosinicella vermicomposti* DSM 21593T; *Sphingosinicella soli* KSL-125T (Yoon et al. 2003), *Sphingomonas paucimobilis* JCM 7516T had a negative nitrate reduction test.

Sulfide-indole-motility (SIM) test

The Sulfide-Indole-Motility (SIM) test used through media to distinguish three parameters, namely Sulfur reduction to distinguish enteric bacteria, Indole test for part of the IMViC test, to distinguish the *Enterobacteriaceae* family, and motility test to distinguish common types of bacteria (MacWilliams 2009). In sulfur reduction, bacterial isolates did not change. According to MacWilliams (2009) bacteria that can reduce sulfur to hydrogen sulfide, then hydrogen sulfide reacts with iron (Iron) to form ferric sulfide, which precipitates black. The indole test on bacterial isolates showed negative results, bacteria that produced a negative reaction on the addition of Kovac's reagent containing HCl, n-amyl alcohol, and p-dimethylaminobenzaldehyde (DMABA) into SIM medium, then p-dimethylaminobenzaldehyde react with indole, producing red Quinoidal compounds (MacWilliams 2009).

Motility is the ability of an organism to move on its own. It was found positive on bacterial isolates isolated in the present study. Motility plays an important role in determining species survival (Miyata et al. 2020; Aygan and Arikan 2007). Almost all spiral bacterial cells and some of the *Bacillus* bacterial cells are motile, while cocci-shaped bacteria are immotile (Aygan and Arikan 2007). This property is caused by the presence of a whip motor device called flagella so that bacterial cells can swim in the water environment (Vadde 2020). The motility of most types of bacteria is motile at relatively low temperatures of 15-25°C and may not be motile at 37°C (Aygan and Arikan 2007). Some bacteria can perform very smooth gliding movements that only occur when in contact with solid objects. Most motile bacteria can approach or move away from various chemical compounds called chemotaxis (Public Health England 2014). All information obtained was by the characteristics possessed by the isolates so that it can be concluded that the isolates came from *Sphingosinicella microcystinivorans*. Based on the research of Qiao et al. (2019) *S. humi* can produce H_2S, but not indole.

Acid production test from glucose

The fermentation media of the isolate showed a green color after incubation for 2 days. The green color indicated that the isolate does not produce acid in glucose fermentation (Public Health England 2019). Isolate A3 has positive characteristics in the oxidation test, Gram-negative bacteria are rod-shaped and do not produce gas and acid in the simple sugar fermentation process. Based on the research of Qiao et al. (2019) on *S. humi* acid produced from D-galactose, but not from D-ribose, lactose, D-xylose, D-sorbitol, D-rhamnose, L arabinose, sucrose, myoinositol,

raffinose, D-fructose, maltose, D-glucose, L- sorbose, mannitol or D-mannose. According to Maruyama et al. (2006), *Sphingosinicella gene* has the major fatty acids are 18:1ω7c and 16:1ω7c. 2-hydroxy fatty acids, with 2-OH 14: 0 predominating. The 3-fatty acid hydroxide is absent whereas. Glycosphingolipids are produced.

hysical properties test

Physical properties test was conducted to determine the environmental factors that affect the growth of bacteria. The results of the physical fat test can be presented in Table 4.

Resistance to NaCl

The results obtained after incubation for 24 hours, namely A3 bacterial isolates can grow well and a lot at the highest salinity of 10% (Table 4). Some microorganisms can survive at high salt or sugar levels, namely osmophilic yeast (growing at high sugar content) (Buzzini et al. 2018) and halophilic bacteria (growing at high salt content) (Moreno et al. 2013). Halophilic bacteria are types of microorganisms that can grow optimally in an environment with high salt concentrations by maintaining osmotic balance (Oren 2008). *Sphingosinicella humi* can grow with 0-0.4% NaCl and grows best without the addition of NaCl and can grow up to 1mM $NaAsO_2$ (Qiao et al. 2007). Based on the capacity of A3 to grow on a medium containing NaCl higher than 2%, A3 does not belong to *Sphingosinicella xenopeptidilytica* (Geueke et al. 2007). Unfortunately, information of *Sphingosinicella xenopeptidilytica* about NaCl tolerance is unavailable from the literature (Maruyama et al. 2006).

Oxygen needs

A3 bacterial isolates after being incubated for 24 hours showed signs of growth on the surface of the tube so that they were aerobic bacteria. Microorganisms require oxygen for growth and their enzyme systems and use O_2 as the final hydrogen acceptor incomplete oxidation changes in higher molecules, such as glucose. Members of the genus *Sphingosinicella* are Gram-negative, rod-shaped, and highly aerobic (Qiao et al. 2007). *S. xenopeptidilytica* has pale yellow colonies, circular and convex. Cells are aerobic and measure 0.6-0.861.5-2.5 mm (Geueke et al. 2007). *S. gene.* have yellow colonies. Truly aerobic and chemo-organotrophic (Maruyama et al. 2006).

Optimum temperature

A temperature test has been carried out to determine the optimal temperature of bacteria, isolate A3 has the optimum temperature at 27°C (Table 4). The optimal temperature usually reflects the normal environment of the microorganism. Temperature plays an important role in enzyme activity. An increase in temperature of 10°C can increase the enzyme activity by two times. At very high temperatures, irreversible protein denaturation occurs, while at very low temperatures, enzyme activity stops. At the optimal growth temperature, there will be an optimal growth rate and the maximum number of cells will be produced (Ji et al. 2021). Each bacterium has an optimal temperature at which they can grow very fast and has a temperature range in which they can grow. The genus *Sphingosinicella* can grow at a temperature of 16-42°C and the optimum temperature is 28°C (Qiao et al. 2007).

Table 4. Results of physical properties of A3 bacterial isolates

Observation Parameter	Result
Resistance to NaCl	10%
Oxygen Needs	Aerobic
Optimum Temperature (^{0}C)	27
Optimum pH	9
Carbon Source	Glucose

Table 5. BLAST results from A3 bacterial isolates

Description	Max score	Total score	Query cover	E value	Per. ident	Accession
Sphingomonadaceae bacterium CBFR-1 16 S ribosomal RNA gne, partial sequence	2503	2503	100%	0.0	99.93%	EF066484. 1
Alpha proteobacterium 7CY gene for 16S ribosomal RNA partial sequence	2503	2503	100%	0.0	99.93%	AB076083.1
Sphingosinicella sp. OC5S gene for 16S rRNA partial sequence	2497	2497	100%	0.0	99.85%	AB429069.1
Sphingosinicella microcystinivorans gene for 16S rRNA partial sequence strain MDB2	2486	2486	100%	0.0	99.71%	AB219940.1
Sphingosinicella xenopeptidilytica strain R25-56 16S ribosomal RNA gene, partial sequence	2477	2477	99%	0.0	99.70%	MN330511.1
Sphingosinicella microcystinivorans B9 DNA complete genome	2481	2481	100%	0.0	99.63%	AP018711.1
Sphingosinicella xenopeptidilytica strain 3-2WA 16S ribosomal RNA, partial sequence	2481	2481	100%	0.0	99.63%	NR_043288.1
Sphingosinicella sp. strain JEZ-8L 16S ribosomal RNA gene. partial sequence	2471	2471	99%	0.0	99.63%	KY406734.1
Sphingosinicella xenopeptidilytica strain LA-50 16S ribosomal RNA gene. partial sequence	2477	2477	100%	0.0	99.56%	MK039097.1
Sphingosinicella microcystinivorans strain Y2 16S ribosomal RNA. partial sequence	2475	2475	100%	0.0	99.56%	NR.040927.1

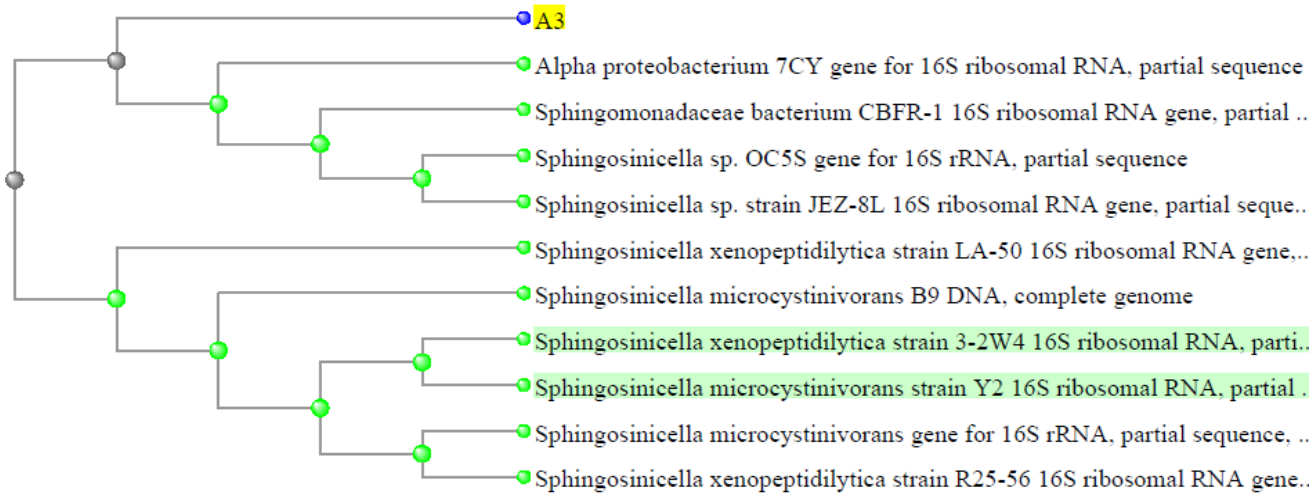

Figure 1. Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, indicating the position of Isolate A3

Optimum pH

The pH tests have been carried out to determine the level of soil acidity, isolate A3 has an optimal pH of 9. The pH is an important for bacterial growth because each bacterial species has an optimum temperature and pH for its growth (Gondal et al. 2021). The pH of the culture medium affects the growth rate, for bacterial growth, there is also an optimal pH and pH range. *Sphingosinicella humi* can grow at pH ranges from 6.5 to 9.0. (Qiao et al. 2007).

Carbon source

Glucose, sucrose, lactose, and maltose are some carbon source used by different bacteria. A3 isolate grew well on glucose carbon sources. Fermentation media must contain compounds that can be oxidized and fermented by microorganisms. To determine the presence of carbohydrate fermentation, simple sugars in the form of glucose, sucrose, lactose, mannitol, and maltose are used. 10% carbohydrate compounds in the form of glucose, sucrose, lactose, maltose, and mannitol, were sterilized separately from the medium. The use of carbohydrates as a carbon source was characterized by the formation of acid which was indicated by a change in the color of the medium to yellow.

Genotypic characterization

DNA isolation was only carried out on A3 isolates as they had good growth in mercury-treated media. The primer used was a 16S rRNA primer with a primary base sequence of 27 F (AGAGTTTGATCMTGGCTCAG) and 1492 R (TACGGYTACCTTGTTACGACTT) (Stackebrandt and Goebel 1994). The 16S rRNA primer is a universal primer that can identify various kinds of bacteria by molecular method. The initial stage of this identification process is the DNA isolation process using the alkaline lysis method, and then followed by the amplification process by PCR to the electrophoresis process. The result of the 1kb ladder electrophoresis marker showed that the DNA band of A3 isolates was 1358 bp. The results of the amplification that have shown a band measuring 1358bp are then continued for the final process of purification and sequencing. After getting the results of the sequencing of bacteria, the bioinformatics process is continued. Table 5 was obtained from the BLAST program at www.ncbi.nih.nlm.gov.

Based on Table 5, it is known that isolate A3 has a very close relationship with *Sphingosinicella microcystinivorans* strain Y2^T. The nucleotide similarity rate of about 80% belongs to the high similarity level (Addinilia 2012). To confirm the position of the isolates in the phylogeny, a number of representative strains of *Sphingosinicella* were selected for the construction of the phylogenetic tree, using *Alpha proteobacterium* as the out-group, see Figure 1.

Based on Figure 1, it is known that the position of Isolate A3 is closely related to *S. microcystinivorans* strain Y2^T. The genus *Sphingosinicella* belongs to the family Sphingomonadaceae in the *Alphaproteobacteria* class. *S. microcystinivorans* Y2^T was first discovered by Maruyama et al. as microcystin-degrading bacteria (Maruyama et al. 2006; Qiao et al. 2007). Microcystins (MCs) are toxins produced by cyanobacteria, photosynthetic microorganisms that preferentially inhabit aquatic environments (Bittencourt-Oliveira et al. 2014). So far, there are only four species of *Sphingosinicella* including *S. microcystinivorans* (Maruyama et al. 2006), *S. xenopeptidilytica* (Geueke et al. 2007), *S. soli* (Yoon et al. 2008) and *S. vermicomposti* (Yasir et al. 2010) represented by strains isolated from eutrophic lakes, wastewater aeration tanks, alkaline soils, and vermicompost. The characteristics of members of the genus *Sphingosinicella* include Gram-stain-negative, rod-shaped and highly aerobic (Maruyama et al. 2006). This is in accordance with the characteristics of the isolate A3.

ACKNOWLEDGEMENTS

The authors would like to express deepest gratitude to reviewers for their patient guidance, useful critiques, advice, and enthusiastic encouragement to this research.

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