Isolation and Identification of *Desulfovibrio* sp. Bacteria from Acid Sulfate Soil

Nur Anny Suryaningsih Taufieq[1,*] , Sahibin Abdul Rahim [2,3], Habibah Jamil [2], Huyyirnah[4] and Amal Arfan [5]

[1] Department of Civil Engineering and Planning, Faculty of Engineering, State University of Makassar, 90224, Parangtambung Makassar, South Sulawesi, Indonesia

[2] School of Environmental Science and Natural Resources, Faculty of Science and Technology, The National University of Malaysia,43600, Bangi, Selangor DE, Malaysia

[3] Research Center for Tropical Climate Change System, Faculty of Science and Technology, The National University of Malaysia,43600, Bangi, Selangor DE, Malaysia

[4]Faculty of Marine Sciences and Fisheries, Hasanuddin University, 90245, Tamalanrea Makassar, South Sulawesi, Indonesia

[5] Department of Geography, Faculty of Mathematics and Natural Science, State University of Makassar, 90224, Parangtambung Makassar, South Sulawesi, Indonesia

[*]*Corresponding author's email: nurannytaufieq [AT] yahoo.co.id*

ABSTRACT—*The purpose of this research is to acquire sulfate reducing bacteria (SRB) is used for bioremediation of acid sulfate soils. The method used in this isolation is pour plate method in the medium Desulfovibrio Medium with Lactate under anaerobic conditions. This method consists of dilution stage with physiological saline solution (NaCl 0.9 percent) sterile, inoculation stage in the liquid agar media, testing growth of bacteria isolates on medium Bismuth Sulfite Agar (BSA) with scratches methods, Gram stain test, H_2S test and sequencing DNA. Results of the test isolates growth in medium Bismuth Sulfite Agar (BSA) showed bacterial isolates produced have breed well. Gram stain results SRB bacteria isolates showed red cells (Gram-negative) and rod shaped bacillus. H_2S test results at Triple Sugar Iron Agar (TSIA) media showed positive results (the formation of H_2S). The amplified DNA using the 16S rRNA primers in PCR showed a band of 1324 bp. The analysis of 16S rRNA sequences using BLASTN program shows that bacteria produced has 84% sequence similarity with Desulfovibrio sp..*

Keywords— acid sulfate soils, *Desulfovibrio*, isolation, pour plate method and SRB

1. INTRODUCTION

Isolation of bacteria in this study is meant to obtain sulfate reducing bacteria (SRB) that is used for bioremediation of acid sulfate soils. Isolation is done by using an agar media as bacteria growing media. Literature review cited one method that has been successfully used to obtain the bacteria is pour plate method. The principle of this method is when there a surviving microorganism cells that were grown in a suitable media, then the cells will proliferate and form colonies that can be viewed directly and counted after incubation performed at a certain temperature and time (Djide & Sartini 2008).

Previous research report includes the isolation of SRB from Tunisian marine sediments using the Hungate technique in the basal medium (Haouari et al. 2006). This technique follows the rules of Widdel & Pfennig (1981). The characterization result using this technique is consistent with members of the genus *Desulfovibrio* that is vibrio-shaped, motile, Gram-negative, non-spore forming rods and positive for desulfoviridin. In addition, new species of *Desulfovibrio* that is *Desulfovibrio bizertensis sp*. *nov*. is also found.

Das & Qin (2012) have done the isolation of cattle rumen (*Bos taurus*) using conventional techniques of serial dilutions on specific media for rumen bacteria in anaerobic conditions. The result identified two isolates of *Butyrivibrio fibrisolvens* that are *Streptococcus* species and *Clostridium aminophilum.* Fichtel et al. (2012) have done the isolation of SRB from sediments above the deep-subseafloor aquifer using agar media dilution method with added artificial seawater according to the rules of Widdel & Bak (1992) and Balch et al. (1979). Strains affiliated with *Desulfosporosinus lacus*, *Desulfotomaculum* sp., and *Desulfovibrio aespoeensis* were isolated only from the top layers (1.3–9.1 meters below

seafloor, mbsf), whereas several strains of *Desulfovibrio indonesiensis* and a relative of *Desulfotignum balticum* were obtained from near-basement sediments (240–262 mbsf).

The most important sulfate reducing bacteria are *Desulfovibrio sp.* and *Desulfomaculum sp*. Benedetto et al. (2005) stated that sulfate reducing bacteria can be used to reduce the acidity of soil by reducing sulfate into sulfur. The study of Fichtel et al. (2012) showed that *Desulfovibrio aespoeensis*, *Desulfovibrio indonesiensis*, and *Desulfotignum balticum* indicated that all reduce sulfate with a limited number of short-chain *n*-alcohols or fatty acids and were able to ferment either ethanol, pyruvate, or betaine. This study aims to report the guideline to isolation *Desulfovibrio sp.* bacteria from acid sulfate soil.

2. MATERIALS AND METHODS

2.1 Acid Sulfate Soil

Composite soil samples were taken from soil layers precisely between 20-30 cm in Rampoang Village, Malangke District, North Luwu, South Sulawesi, Indonesia, at latitude 01°53'19"- 02°55'36"N and longitude 119°47'46"-120°37'44"E. The soil samples were dried at room temperature, pulverized, and then sieved with a mesh 9 sieves. Chemical characteristics of the soil samples are pH 3.67, C-organic 2.34%, organic matter 4.03%, N-total 0.15%, P-available 14.25 ppm, K-exch 0.12 cmol(+)kg^{-1}, cation exchange capacity (CEC) 10.14 cmol(+)kg^{-1} and soil sulfur-total 2.85 % with C/N ratio of 15.60.

2.2 Isolation of Sulfate Reducing Bacteria (SRB)

Isolation of sulfate reducing bacteria is done by using the medium *Desulfovibrio Medium with Lactate* (Atlas & Park 1993) under anaerobic conditions. The media is sterilized with autoclave at temperature 121°C and two atm pressures for 15 minutes. Media contents are as follows: 10.0 g L^{-1} Na lactate + 1.5 g L^{-1} $MgSO_4.7H_2O$ + 1.5 g L^{-1} Na_2SO_4 + 5.0 g L^{-1} glucose + 5.0 g L^{-1} peptone + 3.0 g L^{-1} meat extract + 0.2 g L^{-1} yeast extract + 0.1 g L^{-1} $Fe(NH_4)_2(SO_4)_2$ + 15.0 g L^{-1} agar. Once all of the materials are dissolved, the pH is adjusted to reach pH 4 by using HCl or KOH.

Prior to isolation, several dilution series was made by placing 10 g of acid sulfate soil into a test tube containing 90 ml of sterile physiological saline solution (NaCl 0.9 %). This solution is further stirred using a vortex for 5 minutes to obtain stock solution. To make a 10^{-1} dilution, exactly 1 ml of stock solution is placed into a test tube containing 9 ml of sterile physiological saline solution, and then stirred with a vortex. From the 10^{-1} dilution, 1 ml of solution is placed into a test tube containing 9 ml of sterile physiological saline solution to obtained 10^{-2} dilution (Widyati 2006). Each tube is added 3 mm layer of paraffin to create anaerobic conditions and made duplicate colonies of bacteria that can be calculated (Zamora & Malaver 2012).

Isolation process is done by taking 1 ml of the desired dilution then inoculated into a sterile petri dish. After that, about 15 ml to 20 ml liquid agar media is added and homogenized gently with circular motion. After the agar media become compact, the isolate is then incubated for 3-7 days in anaerobic jars granted AnaeroGen (Oxoid) for anaerobic incubation at 35 °C. Sulfate reducing bacteria will grow at the bottom most of the test tube used. SRB growth is signified with the appearance of dark brown to black colonies (Djide & Sartini 2008). The result of this isolation is a mixed culture. To isolate the bacteria further, scratches methods on the same medium was used to obtain isolates of SRB pure culture which would be used as test bacteria.

Growth test of the isolates is done on medium *Bismuth Sulfite Agar* (BSA) by scratches methods and incubated for 48 hours anaerobic jars granted AnaeroGen (Oxoid) for anaerobic incubation at 35°C. Scratches method is quite difficult for beginners. The principle of this method is to get a bacteria colony that is completely separated from the other colonies of bacteria so that the process of isolation is simplified. This method is done by dividing the petri dish into 3-4 parts. Steriled ose is sticked to a source of bacteria isolates then the ose is scratched into a petri dish containing sterile media. Scratches can be done 3-4 times forming horizontal lines at one side of the dish. Ose is sterilized again with bunsen flame. After becoming dry the ose is used to scratch the previous scratches on both sides of the dish. This step is repeated until all four sides of the petri dish are scratched. In this method, the first scratch is expected to have solid and squeezed growth of bacteria colonies, whereas in the second side scratches bacteria colonies may appear sparsely and so is the next. The whole stage of the experimental procedure is done aseptically so that no contamination may occur. The final colour of the bacteria isolates obtained, appears in black on a medium (Kurokawa 2013).

2.3 Gram Stain Test

Gram stain test is performed to the final isolated bacteria with gram test solution as follows:

a. Hucker's ammonium oxalate crystal violet
A solution: 2.0 g crystal violet + 20.0 ml ethyl alcohol (95%)
B solution: 0.8 g ammonium oxalate + 80.0 ml H_2O
Solution A and B is mixed, and left for 24 hours before use, after which it was filtered into storage bottle.

b. Lugol's Iodine (Gram's modification)
1.0 g Iodine (I_2) + 2.0 g potassium iodide (KI) + 300.0 ml H_2O
Iodine solution is left to dissolve for a few hours or one night in a dark bottle or refined the Iodine and KI in a mortar, with addition of water slowly. Continue to refine I and KI in the mortar while rinsing with remaining water. The refined I and KI is stored in a dark bottle.

c. Incomparable coloring
Stock solution: 2.5 g safranin O + 100.0 ml ethyl alcohol
Working solution: 10.0 ml stock solution + 90.0 ml aquades

The initial step for gram staining is to make a thin smear of the suspension from pure colonies of bacteria aged 24 hours on a clean glass object, then air dried. After dry, the colony is fixed on the glass by passing the bottom of the glass object on the bunsen flame. After that, the bacteria smear is flooded with crystal violet solution for 1 minute, rinsed with running water for a few seconds, then air dried. The air dried bacteria smear is later flooded with iodine solution and left to stand for 1 minute, before rinsing with running water for a few seconds and air dried. The bacteria smear is further rinsed with alcohol for 30 seconds and then air dried. Flushing with alcohol (95%) should not be too long to avoid the washing off of the absorbed dye by Gram-positive bacteria. The next step is to rinse the bacteria smear with running water for 2 seconds and then flooded with safranin for 10 seconds. Further rinsing is done with running water quickly then air dried and observed the results of gram staining under compound microscope with 1000x magnification using emersion oil. Cells of Gram-positive bacteria are stained purple to dark blue whereas Gram-negative bacteria are red. The bacteria found in the growth media is further subjected to biochemical tests which include H_2S test, catalase test, indole test, and urease test.

2.4 H_2S Test

A bacteria inoculum from a pure culture is scratched to the surface of *Triple Sugar Iron Agar* (TSIA) media. After that, TSIA media are incubated for 24 hours at 35 °C - 37 °C and the results are determined. Three types of sugars are found in TSIA media which are 0.1% glucose, 1% sucrose and 1% lactose. If any of the sugars can be used by bacteria as a food source, it will form hydrogen sulfide (H_2S). Hydrogen sulfide (H_2S) may react with iron (Fe^{2+}) to form FeS compounds that appear as black colour in TSIA media indicating a positive test result.

2.5 PCR (Polymerase Chain Reaction)

PCR was performed using specific primers Bacteria 16S rRNA. PCR requires several components, namely: GoTaq MasterMix Green 25 µL, 20 µM Primer 16S rRNA Forward 1 µL, 20 µM Primer 16S rRNA Reverse 1 µL, ddH_2O 18 µL and DNA Template 5 µL. PCR was performed using machines PCR Applied Biosystems Veriti Thermal Cycler 96 Well (Fig. 1).

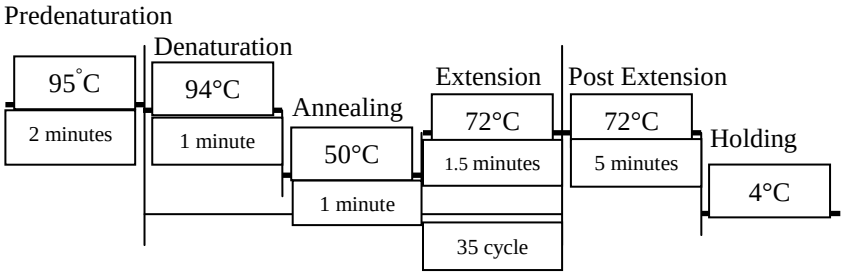

Figure 1. PCR Applied Biosystems Veriti Thermal Cycler 96 Well condition

2.6 Electrophoresis

Electrophoresis was performed with entering 6 µL of sample into each well on 1.5% agarose gel with 1x TAE buffer and run at the condition 100 V for 60 minutes. Gel electrophoresis results visualized by UV to see the band of the amplified DNA.

2.7 DNA Sequencing

Band of the amplified DNA is sent for sequencing. Two nucleotide sequences obtained were examined for related sequences derived from GenBank database with BLASTN program (Altschul et al. 1990).

3. RESULTS AND DISCUSSIONS

Isolation of sulfate reducing bacteria can be done by several methods one of them is pour plate method. In this method, the required treatment before dilution was inoculated into agar medium in a petri dish (Djide & Sartini 2008). Dilution aim is to inoculate microorganisms suspension contained in the petri dish so that less possibility of other organisms inclusion (Saida 2001). Dilution is usually done in decimal of 1: 10, 1: 100, 1: 1000 (10^{-1}, 10^{-2}, 10^{-3}) and so on. As a diluting solution sterile water, physiological saline sterile solution (NaCl 0.9 percent), Ringer solution or phosphate buffer solution can be used (Djide & Sartini 2008). Isolation of bacteria with this method is determined by the accuracy the media pouring at the time of isolation (Fig. 2, 3, 4 & 5).

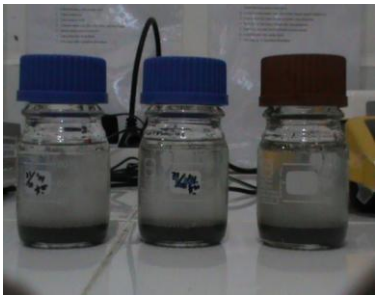

Figure 2. Stock solution

Figure 3. SRB isolation process

Figure 4. SRB incubation process

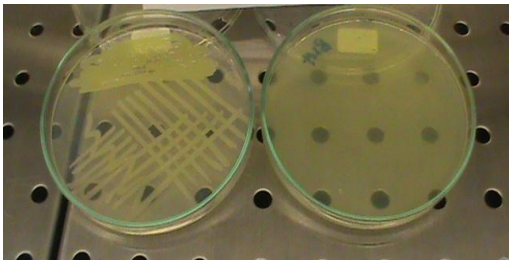

Figure 5. SRB isolate pure cultures

After acquiring the SRB pure cultures isolate, it is necessary to test the growth of isolates on *Bismuth Sulfite Agar* (BSA) medium. According Djide & Sartini (2008), in order for the bacteria to be apparent, the bacteria should be grown in a specific media such as solid nutrient agar media or other selective media so that the properties of colonies can be observed. For SRB, the growth media used is a *Bismuth Sulfite Agar* (BSA) medium. The result obtained is a black bacteria isolates in the medium indicating that bacteria can grow well (Fig. 6).

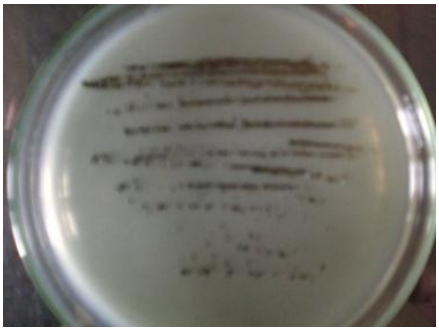

Figure 6. Bacteria isolate in *Bismuth Sulfite Agar* (BSA) medium

Once the process of isolation is complete, it is needed to be tested to determine the bacteria that are found. One type of test that can be done is the Gram reaction test. Gram reaction test is the initial stage in identifying bacteria. This testing is done to differentiate bacteria into two large groups based on the structure of the bacteria cell wall of Gram-positive bacteria and Gram-negative bacteria (Fig. 7). According to Pelczar & Chan (1988), the structure of the cell wall of Gram-negative bacteria is more complex than the structure of the cell wall of Gram-positive bacteria. Gram-negative bacteria have a cell wall composed of three layers namely the outer layer, the middle layer, and the inner layer. The Gram-positive bacteria have only a single layer on the walls of his cell.

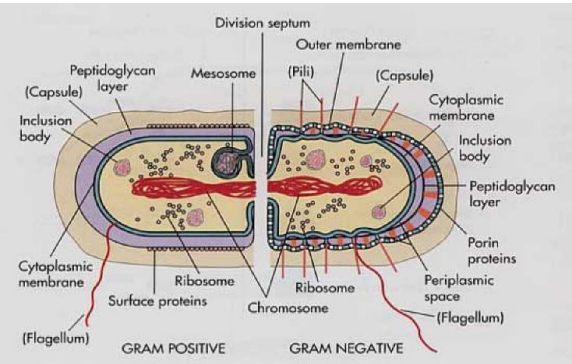

Figure 7. Difference bacteria cell walls of Gram-positive and Gram-negative
(Badan Karantina Pertanian 2008)

Gram reaction test is performed by Gram staining method. In this method apart from knowing the Gram reaction of bacteria culture, it also can be observed the shape and size of cells. Gram-positive bacteria are purple, while Gram-negative bacteria are red when observed under the compound microscope 1000x magnification (Fig. 8).

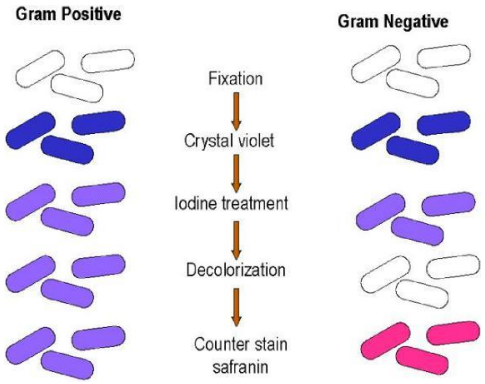

Figure 8. Gram Stain reaction test (Ekmonsaurus 2008)

SRB bacteria isolates of Gram stain result showed red cells (Gram-negative) and rod shaped bacillus (Fig. 9). The number of bacterial colonies is 7.39 x 10^2 CFU mL^{-1}. According to Saida (2001), sulfate reducing bacteria (*Desulfovibrio* sp.) have a spiral to *vibrioid* shaped cells, measuring 0.5 to 1.3 units x 0.8 to 5.0 µm, and are highly anaerobic.

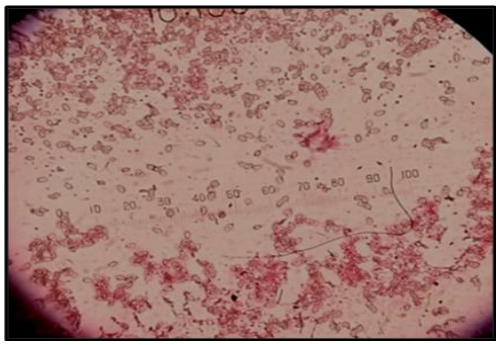

Figure 9. SRB isolates: basil shape, Gram-negative

After Gram stain test, further H_2S test is done to see if the bacteria found can reduce sulfur into sulfide during the process of metabolism. Test results at TSIA media showed positive results because the surface of media is black which means that formation of H_2S occurred (Fig. 10).

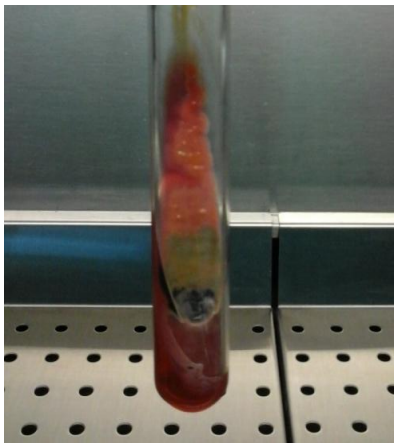

Figure 10. H_2S test result shows bacteria isolated is black

The amplified DNA using the 16S rRNA primers in PCR showed a band of 1324 bp. (Fig. 11). The analysis of 16S rRNA sequences using BLASTN program shows that bacteria produced has 84% sequence similarity with *Desulfovibrio* sp. (Fig. 12) with electropherogram in Fig. 13.

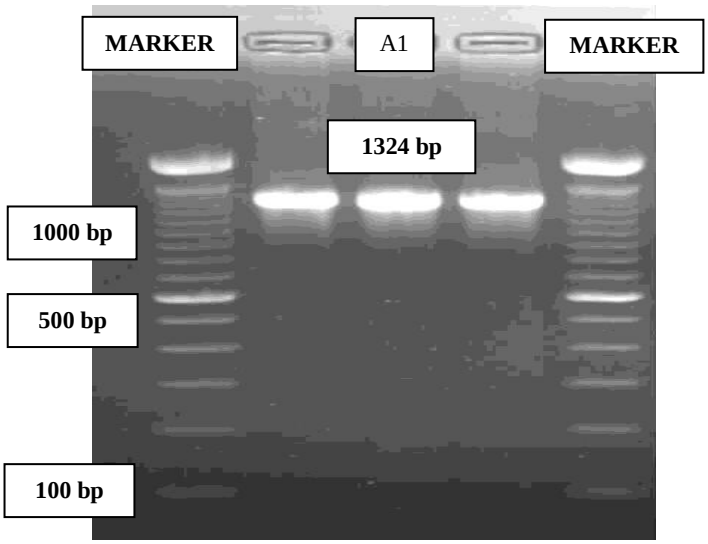

Figure 11. The results of DNA amplification using 16S rRNA primers in PCR

Figure 12. The results of sequencing 16S rRNA sequences using BLASTN program

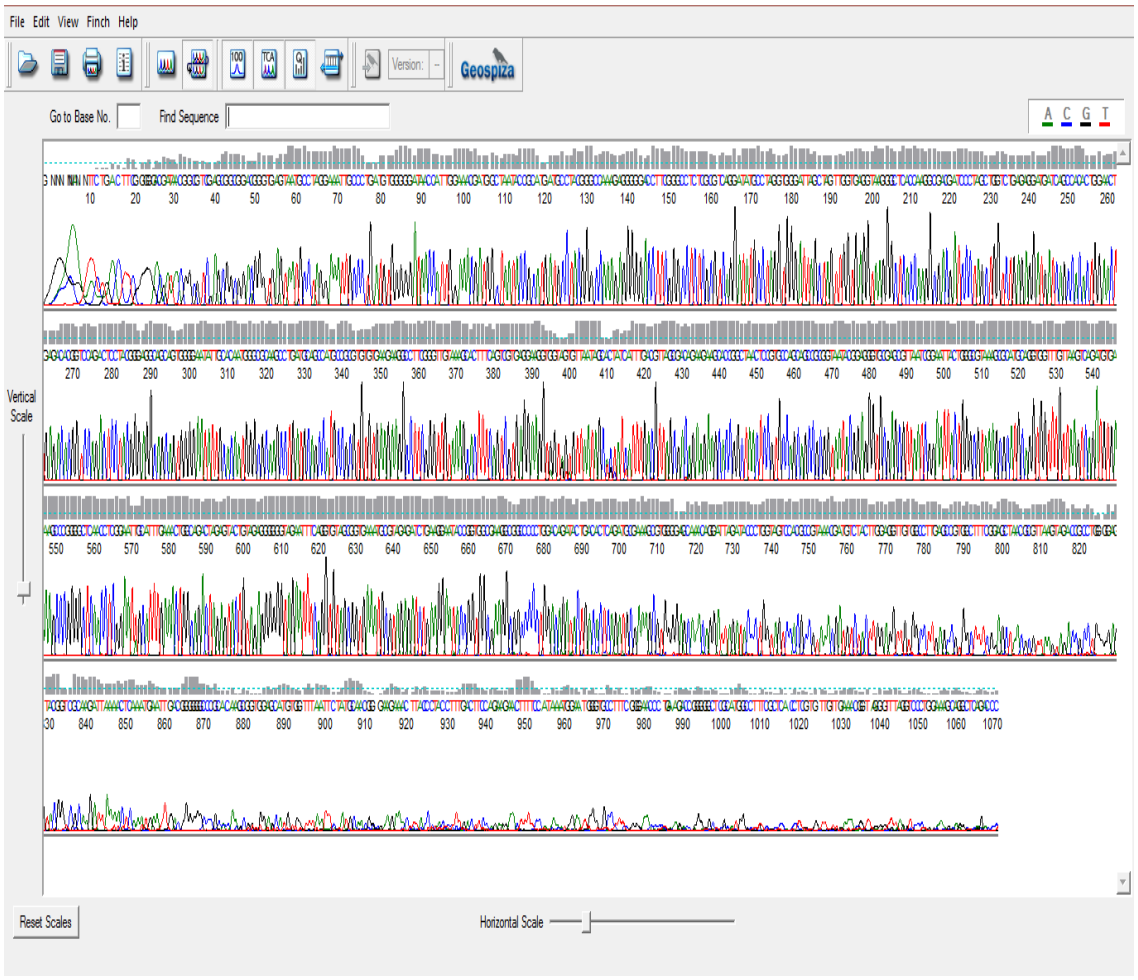

Figure 13. DNA electrophoregram *Desulfovibrio* sp.

Physiological characteristics of the bacteria showed that it is a member of the genus Desulfovibrio. This is supported by analysis of 16S rRNA sequence of 1324 bp including members of the phyla Proteobacteria within the family Desulfovibrionaceae. *Desulfovibrio* sp. is anaerobic bacteria phylogenetic and sulphate reducing bacteria are generally found in soil and aquatic environments. These bacteria are also responsible for the toxic gas hydrogen sulfide in marine sediments and soil environments (Wang &Wise 2015).

Desulfovibrio sp. is a gram negative bacterium, motile, vibrio-shaped, anaerobic, which live in the terrestrial and marine habitats. *Desulfovibrio* sp. contains desulfoviridin and can grow in medium sulphate-lactate in the absence of oxygen as a result of the nature of the metabolic evolution (Sahrani et al. 2008). Based on the use of selective medium *Desulfovibrio* and sodium lactate as a carbon source, Gram stain test and H2S tests that have been conducted, it was concluded that the bacteria are obtained from groups of *Desulfovibrio* sp.. All types of *Desulfovibrio* sp. produce H_2S and desulfoviridin positive, except *Desulfovibrio piger* was motile (Warren et al. 2005). Based on PCR and DNA sequencing, the *Desulfovibrio* found in this research is *Desulfovibrio* sp..

4. CONCLUSIONS

The isolation of SRB using pour plate method was successfully carried out. With this method the SRB pure cultures isolate tested for their bacteria growth on *Bismuth Sulfite Agar* (BSA) media resulting in a black bacteria isolates in the medium indicating that bacteria can grow well. Gram stain test performed on the bacteria isolates yielded red cells (Gram-negative) and rod shaped bacillus. Test of TSIA media showed positive results indicated by the presence of black media surface which designated the formation of H_2S. Based on PCR and DNA sequencing, the *Desulfovibrio* found in this research is *Desulfovibrio* sp..

5. ACKNOWLEDGEMENTS

The first author would like to thanks Department of Civil Engineering and Planning, Faculty of Engineering, State University of Makassar, Indonesia, for the award of study leave and Governor of South Sulawesi for the scholarship to continue her study in the Faculty of Science and Technology, Universiti Kebangsaan Malaysia. Thanks are also due to

Faculty of Science and Technology, Universiti Kebangsaan Malaysia for the use of laboratory and other infrastructure. Most of the laboratory work presented in this paper was carried out in the Department of Marine Science, Faculty of Marine Science and Fisheries, University of Hasanuddin, Indonesia.

6. REFERENCES

[1] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. 1990. Basic local alignment search tool. *Mol. Biol.* 215: 403-410.
[2] Atlas, R.M. & Parks, L.C. 1993. *Handbook of Microbiological Media*. Boca Raton: CRC Press.
[3] Badan Karantina Pertanian Departemen Pertanian. 2008. *Pedoman Diagnosis Optik Golongan Bakteri*. Bogor. pp. 19-32.
[4] Balch, W.E., Fox, G.E., Magrum, L.J., Woese, C.R. & Wolfe, R.S. 1979. Methanogens: reevaluation of a unique biological group. *Microbiol Rev.* 43: 260–296.
[5] Benedetto, J.S., de Almeida, S.K., Gomez, H.A., Vazoller, R.F. & Ladeira, A.C.Q. 2005. Monitoring of sulfate-reducing bacteria in acid water from uranium mines. *Journal Minerals Engineering* 18: 1341-1343.
[6] Das, K.C. & Qin, W. 2012. Isolation and characterization of superior rumen bacteria of cattle (*Bos taurus*) and potential application in animal feedstuff. *Journal of Animal Sciences* 2 (4): 224-228.
[7] Djide, M.N. & Sartini. 2008. *Analisis Mikrobiologi Farmasi*. Cetakan Kedua. Makassar: Fakultas Farmasi Universitas Hasanuddin.
[8] Ekmonsaurus. 2008. Gambar teori pewarnaan bakteri. http://ekmonsaurus.blogspot.com. Diakses pada tanggal 15 Desember 2008.
[9] Fichtel, K., Mathes, F., Könneke, M., Cypionka, H. & Engelen, B. 2012. Isolation of sulfate-reducing bacteria from sediments above the deep-subseafloor aquifer. *Frontiers in Microbiology* Vol.3, Article 65.
[10] Haouari, O., Fardeau, M., Casalot, L., Tholozan, J., Hamdi, M. & Ollivier, B. 2006. Isolation of sulfate-reducing bacteria from Tunisian marine sediments and description of Desulfovibrio bizertensis sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 56: 2909–2913.
[11] Kurokawa, K. 2013. Dasar-dasar mikrobiologi. http://tips-trik-cara-untuk.blogspot.com/2012/04/ dasar-dasar-microbiolog. Diakses pada tanggal 4 Juli 2013.
[12] Pelczar, M.J. & Chan, E.S.C. 1988. *Dasar-Dasar Mikrobiologi*. Edisi 2. Terjemahan Ratna Siri H, Teja Imas, S. Sutarmi, dan Sri Lestari A. Jakarta: UI-Press.
[13] Sahrani, F.K., Ibrahim, Z., Yahya, A. & Aziz, M. 2008. Isolation and identification of marine sulphate-reducing bacteria, *Desulfovibrio* sp. and *Citrobacter freundii* from Pasir Gudang, Malaysia. Sains Malaysiana 37 (4): 365–371.
[14] Saida. 2001. Karakterisasi dan ujian aktivitas isolat bakteri pereduksi aulfat asal ekosistem Air Hitam, Kalimantan Tengah. Tesis. Program Pascasarjana Institut Pertanian Bogor.
[15] Wang, L. & Wise, M.J. 2015. Glycogen with short average chain length enhances bacterial durability. http://www.uniprot.org/proteomes/UP000009173. Diakses pada tanggal 9 April 2015.
[16] Warren, Y.A., Citron, D.M., Merriam, C.V. & Goldstein, E.J.C. 2005. Biochemical differentiation and comparison of *Desulfovibrio* species and other phenotypically similar genera. *Journal of Clinical Microbiology* 43 (8): 4041–4045.
[17] Widdel, F. & Pfennig, N. 1981. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. I. Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov., sp. nov. *Arch Microbiol* 129: 395–400.
[18] Widdel, F. & Bak, F. 1992. Gram-negative mesophilic sulfate-reducing bacteria, in *The Prokaryotes*, eds A. Balows, H.G. Trüper, M. Dworkin, W. Harder & K.H. Schleifer. New York: SpringerVerlag, 3352–3378.
[19] Widyati, E. 2006. Bioremediasi tanah bekas tambang batubara dengan *sludge* industri kertas untuk memacu revegetasi lahan. Disertasi. Sekolah Pascasarjana Institut Pertanian Bogor.
[20] Zamora, A.C. & Malaver, N. 2012. Methodological aspects for the culture and quantification of heterotrophic sulfate-reducing bacteria. *Revista de la Sociedad Venezolana de Microbiología* 32: 121-125.