Eco. Env. & Cons. 27 (1) : 2021; pp. (282-287)
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ISSN 0971–765X

Isolation and characterization of sulfate reducing bacteria from cultivation pond sediments

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(Received 20 July, 2020; accepted 20 August, 2020)

ABSTRACT

Sulfate reducing bacteria (SRB) are bacteria that play a role in the bioremediation process. These bacteria are commonly found in anoxic environment, especially at the bottom of the sediments. This study aims to isolate and characterize sulfate reducing bacteria from cultivating pond sediments. A total of 20 bacterial samples were isolated from pond ecosystems in the Kuri area of Maros Regency, South Sulawesi, Indonesia. Growth of sulfate reducing bacteria was conducted using selective media postgate B by observing the change in color of the media to black. Purification is done by observing the colonies that grow in the media Nutrient Agar (NA) after incubation for 24 hours. Characterization tests include observations of colony morphology and bacterial cells, as well as biochemical tests. The results showed that SRB has four best isolates in sequence, 3A, 3B, 3D, and 4D. Based on the results of the characterization test using profile matching isolate 3A, it was identified as a member of genus *Desolfobulbus*.

Key words : *Sulfate reducing bacteria, Isolation, Characterization, Morphology*

Introduction

Sulfate reducing bacteria (SRB) are obligate anaerobic bacteria living in ocean, rice fields and inland waters. This bacterium uses hydrogen or sulfate as electron acceptor or donor in its metabolic activity. The SRB can reduce sulfate under anaerobic conditions to sulfide or a form of sulfur, precipitating toxic metals (Cu, Zn, Cd) as metal sulfides. Furthermore, under anaerobic conditions, the availability of sulfate in the soil would be limited mainly to soil pH above 5.5. The SRB requires organic substrates derived from short chain organic acids such as pyruvic acid. According to Brierley and Brierley (1997), sulfate-reducing bacterial groups are widely used in controlling sulfates and heavy metals in polluted soils. The SRB belonging to the genus *Desulfovibrio* and *Desulfotomaculum* oxidize organic compounds and hydrogen sulfide using sulfate as electron acceptors to produce bicarbonate (Brierley and Brierley, 1997).

The application of SRB plays an important role in reducing the content of pollutants in the environment characterized by changes in pH, and C-Organic. The SRB is able to restore the acidity of water bodies close to neutral (pH 6-7) and reduce the content of heavy metals dissolved in the waters. This is effectively used in the bioremediation process of exmining land because it is very helpful in rehabilitating ex-mining land. The effectiveness of BPS in-

creases with the presence of organic matter in the environment (Widyati, 2007).

The SRB may grow and colonizein wide range of habitat. These species are most commonly found in marine sediments because its high sulfate content (Widdel and Bak, 1992). In addition to the ocean, these bacteria are also found in inland waters. Postgate and Campbell (1966) and Doshi (2006) suggested the high number of SRB found in lake deposits and inundated land, cattle rumen, geothermal. This can also develop in rice fields, paper mills and rivers that are affected by mine acid waste or water. Since these bacteria are obligate anaerobic bacteria, the SRB are more commonly found in anoxic environments, especially at the bottom of sediments. Jorgensen (1982) reported that the amount and activity of sulfate reducing bacteria increased with the thickness of the sedimentary layer. However, there is a few of the SRB able to grow under oxidic conditions. Hence, the diversity of bacteria that grows in sediments is high. Risatti *et al.* (1994) suggested that the *Desulfo vibrio* sp. more dominant at the top of the sediment, whereas *Desulfotomaculum* sp. found mostly at the bottom of sediments.

Tambak or cultivating pond is located in coastal areas build by a dike to keep fish (especially milkfish; shrimp). According to Boyd (2003), an increase in organic matter at the bottom of the water may cause the least dissolved oxygen, because the increase in oxygen consumption is greater than the level of dissolved oxygen production. This can cause the anoxic layer to get thicker, thereby affecting oxygen loss. In addition, loss of oxygen in water is also caused by oxygen being used by microbes to oxidize organic matter. Immersed pond conditions cause the pond to be anaerobic (without oxygen), thus allowing the SRB to survive. The role of decomposing bacteria in optimal nutrient recycling such as pH, temperature and organic matter. This oxidation process is carried out by anaerobic bacteria. However, decomposing bacteria have limited ability to decompose organic or inorganic materials.

To culture SRB requires a multiplying media containing certain hydrogen donors, assimilated substrates, minerals and sulfates, anaerobic conditions and a sufficiently low redox potential (E'0 = -200 mV) must be sought. To study the life and diversity of bacteria, an effort is needed to culture them on a laboratory scale. This breeding is done by growing bacteria from isolate sources, such as soil, air, food scraps, etc., in enriching nutrient media. Bacterial growth media are very diverse, ranging from selective media, fertilizing media, differential media and others. This study aims to isolate and characterize sulfate reducing bacteria from pond sediments.

Materials and Methods

Bacterial Isolation

The Bacteria were isolated from two cultivating pond of milkfish in the Kuri area of Maros Regency, South Sulawesi, Indonesia. The 10 anoxic sediment samples were collected from each pond sediments, each consisted of 100 - 150 g. These were put into dark colored bottles and packed in anaerobic conditions. The initial phase of isolation is done by diluting the sample in a saline physiological solution, 10% NaCl solution. Dilution was carried out by 10^{-1}, 10^{-2}, 10^{-3}, while the dilution was done by transferring 1 mL of sediment sample into a screw tube containing 9% NaCl as much as 9 mL (10^{-1} dilution). Then for a 10-2 dilution done by taking the results of 10^{-1} dilution of 1 mL into a screw tube containing 10% NaCl as much as 9 mL and from the results of 10^{-2} transferred as much as 1 mL into a screw tube containing 10% NaCl as much as 9 mL then Furthermore, from this dilution grown on sulfate reducing bacteria media, namely postgate media B. The process of culturing sulfate reducing bacteria was done by taking 1 mL of sediment from the dilution that emits the blackest black color in a pipette of 1 mL into a screw tube filled with SRB media. After that, these were incubated in a dark place to avoid the light effect. The observation phase was carried out within 3 days after storage. The growth of SRB was marked by changing the media to black.

Colony Purification and Purification

Purification of the bacteria colony was done by separating or transferring bacterial samples from the liquid media to the Nutrient Agar (NA) medium in order to obtain a pure colony. This stage was begun with the making of NA media on sterilized Petri disk. The amount of 1 ml of the best sample was poured onto the surface of NA petri media, then flattened using Drigalski spatula. Observation of the colony was done after 24 hours of incubation. Colonies that have the same shape were indicated as cultures with one SRB cell type.

Characterization

The bacteria dentification was done by solid and liquid media. The parameters tested were morphology, growth, fluorescent test and yellow colony test. To determine the morphological type, each isolate was crushed on NA petri media then observed the size, shape of the edges, and color of the bacterial colony. Gram reaction was carried out by taking colonies from pure culture on NA media using preparations that have dropped 3% KOH solution. Then slowly leveled, from the results of the gram reaction found negative gram which was marked by a colony that appears slimy.

The fluorescent test was carried out using King's B media. Bacteria that produce positive reactions were marked by a change in color to yellow. The final test at the bacterial identification stage was the yellow colony test on the yeast dextrose carbonate agar media. Positive test results are marked with foam which was seen in bacteria. The identification was carried out in the Center for Research Activities, Hasanuddin University, Makassar, Indonesia.

Results

Isolation and purification

Sulfate reducing bacteria isolated from pond sediments were able to grow and develop on the media. This was evidenced through the test media in the form of liquid SRB media. Bacteria were isolated by dilution using 10% NaCl solution. Diluted solutions were incubated for 3-4 days in a screw tubes with total 60 tubes (20 tubes each from dilution 10^{-1}, 10^{-2}, 10^{-3}). On the fourth day of the incubation stage then the observations of the formed sediment were conducted. The observations showed that the dilution samples at 10^{-2} and 10^{-3} have darker color than those of 10^{-1} so that the 10^{-1} samples were exclude from further test. Hence, the number of propagated samples was 40 screw tube.

After the bacterial isolation step was carried out on the liquid media, an observation was carried out on the third day three times and it was found that out of the 40 tubes grown on the media, there were 12 blackened tubes on the screw tube containing sediment from 10^{-2} dilutions and 8 blackened tubes on a screw tube containing sediment from a 10^{-3} dilution (Figure 1). The four best samples that show the most concentrated black color were, 3A, 3B, 3D, and 4D. those sampled was collected form middle station. A screw tubewith darker color has more SRB, while the gray one indicates that the bacteria was not growing. The color of media change to black indicated of over with sulfate reducing bacteria growth, while those change to gray color indicated no bacteria growth.

Purification of bacterial isolates was based on samples which produced the most concentrated

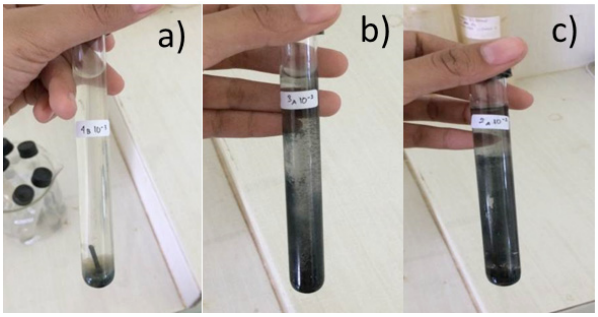

Fig. 1. Color differences in sample colony dilutions a) 10^{-1}, b) 10^{-2}, c) 10^{-3}

black media. The isolate 3A was considered the best sample for further testing. The SRB of 3A isolates were grown on NA petri media. After 24 hours of incubation, the colonies that grew on the media showed the same shape so it was indicated to be from one type of SRB.

Characterization

Purified bacteria were then identified in the laboratory. The initial stage of identification is the morphological characteristics of the bacterial colonies found. As for the results of the bacterial morphological characteristics test obtained were:intact edge, flat leveled elevation, dark beige color and round colony.

The physiological and biochemical characteristic tests were carried out by first conducting a Gram test using 3% KOH. Gram reaction is carried out by taking colonies from pure culture on NA media using preparations that have dropped 3% KOH solution. Then slowly leveled, from the results of the gram reaction found Gram - (negative) which is marked by a colony appear slimy. The results of the SRB characterization test can be seen in Table 1.

Based on the phenotypic characteristics of sulfate-reducing bacterial isolates, all isolates have morphological characters, namely the shape of a rounded colony, intact edges, flat elevation and the color of brownish beige colonies and cell morphol-

Table 1. Genus level identification results based on the Profile Matching method.

Key character	*Desulfo-microbium*	*Desulfobul bus*	*Desulfo-tomaculum*	3A (10^{-2})	3B (10^{-1})	3D (10^{-1})	4D (10^{-1})
Cell morph	Rod shape	Rod shape	Rod shape	Rod shape	Rod shape	Rod shape	Rod shape
Length (µm)	0.6	1.5-2.5	2-9	± 3.48	± 1.65	± 1.69	± 1.62
Sulphide producer	+	+	+	+	+	+	+
Anaerobic	+	+	+	+	+	+	+
Gram	-	-	+	-	-	-	-

ogy, namely short stem and Gram negative (Figure 2). The biochemical test results of SRB isolates showed that it was included in the anaerobic bacteria group. The identification results in Table 1, showed that the isolates were classifiedas genus *Desulfobulbus* because they have the form of short, Gram-negative, sulphideproducer and anaerobic rod cells.

Discussion

The pond sediment collection showed almost entirely containing sulfate reducing bacteria. Pond soils that contain lots of sulfate are suitable habitats for the growth of sulfate reducing bacteria. From the total number of samples, there werefour best SRB isolates. Differences in the ability of bacteria to reduce sulfate was varying. This diversity is caused by environmental conditions, such as pH values, sediment thickness, sulfate content and availability of organic matter (Liamlean and Annachhtre, 2007). Whereas Jorgensen (1982) reported sediment thickness affects the activity and number of bacteria.

Purified SRB is a bacterium that has the highest sulfate reduction ability. This study showed that isolate code 3A was the best isolate because it showed the most concentrated black color. Sulfate reducing bacteria can reduce sulfate in anaerobic conditions to hydrogen sulfide gas (H_2S) to form a black color. Sulfate reducing activity is increasing due to the availability of organic matter. Lens *et al.* (1998) stated that sulfate reducing bacteria require

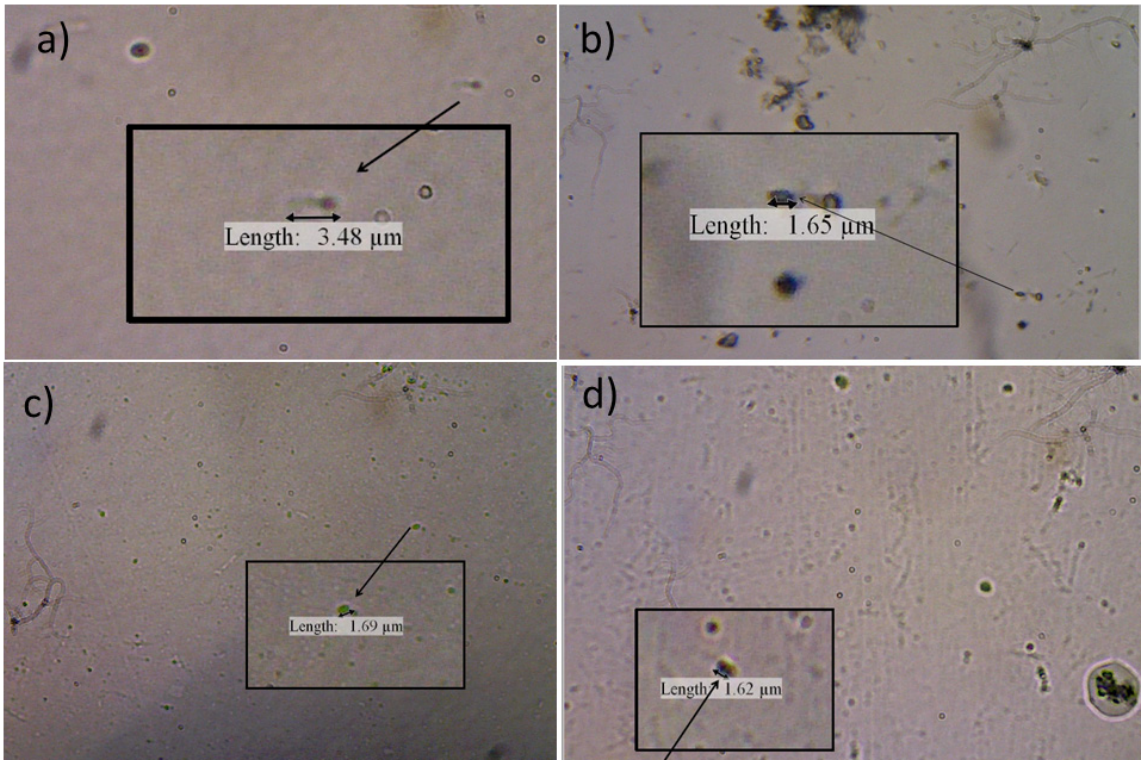

Fig. 2. Morphology of isolates (a) 3A 10^{-2}, (b) 3B 10^{-1}, (c) 3D 10^{-1}, (d) 4D 10^{-1}

electrons from the oxidation activity of organic matter. Sufficient organic matter will support microbial growth because it is used as an energy source and electron donor through the oxidation activity of the organic material. Apart from organic matter, environmental pH, sediment, and sulfate content available in the soil, the depth of the sediment also affected the amount of oxygen contained so that it affects the types and bacteria. Desulfovibrio group was mostly found to appear in upper layer sediments (Rissatti *et al.* (1994).

The pure colony identification stage was carried out using several tests namely morphological, physiological and biochemical characteristics tests. The morphological test was carried out by looking at the bacterial samples that had been etched on the object glass and found the shape of the bacteria was round with an intact edge, brownish-brown in color and had flat elevation (flat surface shape). The Gram reaction was then carried out in physiological and biochemical tests, resulting in the bacteria giving a positive reaction marked by slimy, negative Gram reaction (-). Bacteria produce positive reactions marked by a change in color to yellow.

Sulfate reducing bacteria isolated from our study sited was classified as the genus *Desulfobulbus*. Generally, *Desulfobulbus* strain bacteria grow optimally at temperatures around 30^{o}C and are more common in sediments compared to the Desulfovibrio genus. Barboza *et al.* (2014) found *Desulfobulbus* spp. in pond sediments in Brazil. Likewise, with the results of research from Virpiranta *et al.* (2019) study reported that the common genus SRBin Finland was *Desulfobulbus*.

Sulfate reducing bacteria as an anaerobic obligate bacterium use H_2 as an electron donor (chemolithotrophic). Sulfide-producing was positive marked by the black discoloration in selective media. Sulfate was reduced to sulphide by SRB under anaerobic conditions, then H_2S produced can precipitate toxic metals (Zn, Cu, Cd) as metal sulfides. Pyruvic acid was used by SRB as an organic substrate. Pyruvic acid is produced by other anaerobic activity (Hanafiah *et al.,* 2009).

Sulfate reducing bacteria were effectively used in the bioremediation process of ex-coal mines with an incubation time of 20 days (Widyati, 2007). The administration of sulfate reducing bacteria isolates was also able to help increase acidic sulfate soil pH and increase the growth of corn plants (Sudarno *et al.,* 2018). The reduction process carried out by SRB used sulfate as an energy source, namely as an electron acceptor and uses organic material as a carbon source (C). When sulfates receive electrons from organic matter, they were reduced to form sulfide compounds. Decrease in sulfate concentration will increase soil pH. This occurred due to several interrelated processes, namely because of inundation, addition of organic material and SRB activities (Widyati, 2007).

The sulfate reduction process carried out by microorganisms can be carried out by trapping heavy metals then binding them to the cell wall. This process is called biosorption (Kurniawan and Ekowati, 2016). This mechanism involves several processes, including electrostatic interactions, ion exchange, precipitation, redox processes, and surface complexation. Metal absorption involves covalent and ionic bonds, including proteins and polysaccharides as a source of functional groups that play an important role in binding metal ions. The available ligand groups are negatively charged groups such as phosphates, carboxylates, thiolates or amides and phosphodiester groups that coordinate with the metal's central atom through free electron pairs (Gadd, 1990).

Another bio-removal method was the process by which heavy metal ions pass through the cell membrane into the cytoplasm, through the cycle of cell metabolism, this is called bioaccumulation. This process occurs through the removal of heavy metals which require the activity of living organisms and the energy needed in the process of absorption of metallic cations (Gavrilescu, 2004). This accumulation process can be grouped into bioconcentration and bioaccumulation. *Bradyrhizobium japonicum* was a Gram-negative bacterium that has the potential in bioaccumulation of heavy metals from a polluted environment (Syamsuddin *et al.,* 2005). The presence of heavy metals differs at each trophic level in an ecosystem, depending on the bioaccumulation characteristics of concentrated metals. Heavy metal bioaccumulation occurs actively and is metabolically controlled by organisms. Whereas the heavy metal bioavailability, its accumulation, and its toxicity depend on the variables contained in the environment (Arunakumara and Xuecheng, 2008).

Conclusion

From the results of the study it can be concluded that of the 40 tubes grown on the media, there were

12 blackened tubes in a screw tube containing sediment from a 10^{-2} dilution and 8 blackened tubes on a screw tube containing sediment from a 10-3 dilution. Four best sequential isolates, 3A, 3B, 3D, and 4D, were tested for characterization. Based on the results of characterization tests using profile matching, all isolates were assumed to belong to the genus *Desolfobulbus*.

Acknowledgement

We would like to thank the Ministry of Research Technology and Higher Education (Kemenristekdikti) for funding the research 2017 and enabled the author to publish this article.

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