Vol. 12(21), pp. 492-500, 7 June, 2018
DOI: 10.5897/AJMR2018.8837
Article Number: B819B4357346
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Exploration of sulfate reducing bacteria from polluted waters

Budiarsa Suyasa W.[1]*, Iryanti E. Suprihatin[1], Dwi Adi Suastuti G. A.[1] and Sri Kunti Pancadewi G. A.[2]

[1]Bioremediation Research Division, Udayana University, Denpasar Bali, Indonesia.
[2]Department of Environmental Chemistry, Udayana University, Bukit Jimbaran, Bali, Indonesia.

Received 10 March, 2018; Accepted 14 May, 2018

Sulfate reducing bacteria (SRB) was successfully isolated from Estuary Dam in Suwung Denpasar, Indonesia. This estuary catches highly polluted water from Badung River which runs across and hence carries pollution due to waste disposal from Denpasar City. SRB was studied in detail for their ability to reduce sulfate to sulfide with organic material as an oxidizing agent. SRB exploration of the estuary ecosystem of the contaminated dam was accomplished through isolation, selection and characterization of the isolates obtained. The result of this study found superior SRB named DPS 1711, DPS 1705 and DPS 1703. The bacteria have the ability to grow at pH 3, room temperature and uses compost as organic substrate. This ability is an important factor for the application of isolates in the treatment of acid mine waste. Isolates have optimum optical density under the pH range of 4 to 7 and the best at pH 5 have a growth rate profile at a temperature range of 25 to 40°C. The isolates observed were Gram-negative stem, motile bacteria which only grow in anaerobic condition. Physiological-biochemical characterization showed the three isolates, namely DPS 1703, DPS 1705 and DPS 1711 were SRB groups identified as *Desulfotomaculum orientis*.

Key words: Sulfate reducing bacteria, polluted waters, estuary dam ecosystem.

INTRODUCTION

Estuary Dam Suwung is a lagoon formed at the estuary of the Badung River flowing through the city of Denpasar. Badung River has long experienced pollution due to waste disposal from various activities along its bank. The high pollutants load entering the estuary dam of Suwung Denpasar has led to the decreasing quality of the waters and the formation of oxygen deficit zones at the bottom. Pollution containing high concentration of organic materials tends to cause the acidic and anaerobic conditions of the aquatic sediments. These conditions affect the composition of biological species with biogeochemical cycles. The acidic environment can trigger the formation of reactive metals in the form of their ions, thereby causing metal contamination in the aquatic environment. Meanwhile, sludge from wastewater treatment becomes its own problem with heavy metal content and low pH. This high level contamination of heavy metal requires development of a sludge

*Corresponding author. E-mail: yandiars@gmail.com.

management system to treat the acidity and heavy metal content. Biotechnology has been utilized in waste water treatment. Using this technology, the contaminant is removed through the precipitation of the contaminant in the reduced form by the microbial activity. Furthermore, on a certain scale, the precipitated contaminants can be recovered.

Anaerobic biological processes encourage sulfide formation by sulfur reducing bacteria. SRB is heterotrophic bacteria that use simple organic compounds as carbon sources. With metabolic respiration, the bacteria utilize sulfate, thiosulfate, sulfites and other reducible sulfur compounds as electron acceptor (Müller et al., 2014).

The sulfur in the oxidized state will be reduced to sulfide in the anaerobic environment (Barton and Faugue, 2009). SRB is a true anaerobic microorganism with a primitive respirative pathway capable of living in extreme environments. The group of bacteria is generally isolated from aquatic sediments that have extreme conditions, as temperature, pH, alkalinity, sulfate, iron, manganese, ammonium and phosphate contents (Rückert, 2016). This research aims to isolate and characterize SRB from water sediments contaminated with domestic waste.

MATERIALS AND METHODS

Isolation

The soil samples were taken from Estuary Dam in Suwung, South Denpasar area of Bali. There are about 12 sampling points in one area. For SRB exploration, soil samples taken from sediments were immediately inserted into polyethylene bottles and stored in a cooler box for transporting and then stored in a freezer in the laboratory. The number of samples taken at each sampling point ranged from 100 to 200 g of soil. SRB group isolation was based on Postgate medium B liquid media composition (Atlas, 1993). The pH 4 setting was performed with 10% sulfuric acid and 10% sodium hydroxide prior to sterilization. Samples suspended in 0.85% NaCl and diluted to 10^3 were then cultured in an anaerobic tube. The cultures were then incubated at 35°C. Observations were made from the time when color started to change until all turned black. Blacken isolates were then purified in liquid medium (Rückert, 2016). The isolates were suspended and diluted further in the same manner until they reached dilution level of 10^{12}. Growth time of the bacteria was observed from the time of black color appearance until the entire tube turned black. Isolates grown at the final dilution rate were indicated as cultures with one type of SRB cell.

Characterization of SRB cells

Isolates indicated as SRB cells were tested for their ability to reduce sulfate and increase the pH of the media. Isolates were cultured in a liquid medium containing 500 ppm sulfate. Trials were carried out in media with pH variations of 7, 5, 4 and 3. One mL of SRB cells isolate was transferred aseptically into a threaded tube filled with 1/3 liquid medium (500 ppm sulfate with 7, 5, 4, or 3), and filled up with medium, then incubated at 35°C. On the 15th day, the residual sulfate and pH of the solution were measured. The isolates capable of growing at low pH and reducing sulfate (and increasing pH of the media were selected.

Table 1. Isolates growth time.

Sample code	Growth time (days)	Colour intensity
DPS 1701	5	+++
DPS 1702	5	+
DPS 1703	5	++++
DPS 1704	8	++++
DPS 1705	8	+ +++
DPS 1706	9	++++
DPS 1707	5	++
DPS 1708	5	++
DPS 1709	5	+
DPS 1710	7	++
DPS 1711	7	++
DPS 1712	8	+

+: Thin black on the bottom; ++: Black evenly distributed almost all parts of the tube; +++: Black evenly on all parts of the tube; ++++: Solid black evenly across the tube.

RESULTS AND DISCUSSION

Isolation and characterization

The estuary dam from where the samples were collected has undergone siltation, indicated by its blackish brown sediment, low pH (5, 6 and 3) and high pollutant contents. All 12 soil samples isolated with Postgate B liquid media, change from clear to black under anaerobic conditions, which indicate the presence of SRB (Dennis and Julia, 2014). The black color shows the presence of sulfide (reduced sulfur) which is the result of sulfate reduction by SRB (Xu et al., 2013). The growth rate observed by the time required to turn the colour of the suspension black varies widely. The growth time of SRB from the isolated samples is shown in Table 1. The capability of the SRB to reduce sulfate, and therefore increase pH, as measured after 15 days culture, is shown in Table 2. The observation found nine isolates capable of growing at all pH variations, and only three isolates did not grow at pH 3 (Table 2). The pH changes and sulfate reduction efficiency is shown in Table 3. The sulfate reduction efficiency was determined by calculating the percentage of the reduction of the sulfate concentrations. Table 3 shows the sequence of ability to increase pH and sulfate reduction efficiency of nine isolates. High efficiency sulfate reduction is an important factor for sulfide formation associated with pH increase.

Three isolates appear to have a prominent activity compared to the other six isolates. The three isolates namely DPS 1711, DPS 1703 and DPS 1705, are also the ones with highest growth rates (Table 1). Bacterial cells grow rapidly by splitting in the supporting environment. When food is abundant then the term survival of the fittest which implies that, the winning type is the one who can divide the most rapidly. Faster splitting ability allows bacterial populations to adjust immediately

Table 2. pH and sulfate concentrations of the isolates grown at various initial pH and [SO^{2-}] of 500 ppm.

Isolate code	Initial pH 7		Initial pH 5		Initial pH 4		Initial pH 3	
	pH	$[SO_4^{2-}]$(ppm)	pH	$[SO_4^{2-}]$(ppm)	pH	$[SO_4^{2-}]$(ppm)	pH	$[SO_4^{2-}]$(ppm)
DPS 1711	8.1	39.11	7.6	68.32	6.3	78.16	6.1	83.57
DPS 1703	7.9	44.06	7.6	66.35	5.9	83.24	5.8	94. 31
DPS 1705	7.9	48.72	7.4	81.23	5.8	87.05	5.8	91.56
DPS 1701	7.6	88.92	6.5	127.55	5.8	158.54	4.4	188.62
DPS 1710	7.6	93.24	6.3	153.25	5.8	162.37	4.4	192.67
DPS 1706	7.6	91.79	6.3	158.81	5.7	171.28	4.2	197.25
DPS 1704	7.6	101.97	6.2	167.89	5.5	166.37	4.2	267.17
DPS 1707	7.5	124.31	6.0	193.96	4.4	204.36	3.8	285.01
DPS 1708	7.5	122.46	6.0	193.83	4.2	216.54	3.3	326.67
DPS 1702	7.5	129.65	6.0	197.55	4.1	221.42	NA	NA
DPS 1712	7.3	135.28	6.0	200.49	4.0	223.12	NA	NA
DPS 1709	7.3	131.18	5.8	214.03	4.0	226.89	NA	NA
Control	NA	NA	NA	NA	NA	NA	NA	NA

NA: Unmeasured due to unobservable growth.

Table 3. Changes in pH and sulfate reduction efficiency.

Isolate code	pH 7		pH 5		PH 4		pH 3	
	ΔpH	E (%)	ΔpH	E (%)	ΔpH	E (%)	ΔpH	E (%)
DPS 1711	1.2	92.27	2.5	86.45	3.2	84.36	3.7	83.28
DPS 1703	1.0	91.29	2.5	86.84	2.9	83.35	3.3	81.13
DPS 1705	1.0	90.37	2.3	83.89	2.8	82.78	3.3	81.69
DPS 1701	0.8	82.40	2.2	82.64	2.8	68.65	1.9	66.79
DPS 1710	0.7	81.58	2.0	83.49	2.8	66.13	1.9	66.07
DPS 1706	0.7	81.87	2.0	82.39	2.7	66.13	1.3	62.92
DPS 1704	0.7	79.86	1.1	66.71	2.5	67.10	0.8	52.96
Control	NA	NA	NA	NA	NA	NA	NA	NA

NA: Not measured due to unobservable growth.

to changes in the environment (Shiqiang et al., 2014). Microorganisms interact with their environment in a variety of ways. The ability to utilize certain nutrients, producing metabolites that affect other microorganisms and interacting with the physical and chemical environment are the factors that determine the growth of a microorganism. These conditions define the activity and growth character of a species, which is different from the other species (Kato, 2016). The ability to grow at room temperature is a very important factor for the efficient application of SRB isolates in bioreactors. The ten isolates selected in the first selection, were further selected for the purpose of the determination of the reduction efficiency. The tested isolates were cultured on a Postgate B liquid medium containing 1000 ppm sulfate at pH 3 and incubated at room temperature (27 to 30°C).

Measurements and observations of growth time, optical density, pH increase and sulfate reduction different efficiency are shown in Table 4. Measurements of optical density, pH increase and sulfate reduction efficiency at room temperature are performed after a 21 day incubation period with estimated exponential phases being exceeded. Isolates capable of growing rapidly at room temperature, raising the pH of the medium and reducing the sulfate with high efficiency (that is >80%) are selected isolates. The selected isolates are DPS 1703, DPS 1705 and DPS 1711 (Figure 1). Growth at room temperature and activity on acidic medium (low pH) are very important properties in the applications for treating acidic mine waste and are important factors for the efficiency of the bioreactor performance.

Cell morphology and physiology

Observations under microscope of simple staining, Gram staining, and spores to determine cell morphology were performed on all three selected isolates. Physiological

Table 4. Growth time, pH increase, OD, and sulfate reduction of 7 isolates at pH 3 and room temperature.

Isolate code	Growth time (days)	Optical density	ΔpH	Sulfate reduction ppm	Efficiency (%)
DPS1711	8[a]	0.671[a]	4.3[a]	831.28[a]	83.13[a]
DPS 1703	7[a]	0.650[b]	4.2[a]	829.32[a]	82.92[a]
DPS 1705	8[a]	0.602[c]	3.9[b]	805.24[b]	80.52[b]
DPS1706	11[b]	0.421[d]	2.5[c]	613.68[c]	61.37[c]
DPS 1701	12[b]	0.387[e]	2.3[d]	556.83[d]	55.68[d]
DPS 1710	15[c]	0.382[e]	2.1[e]	547.73[e]	54.77[e]
DPS 1704	15[c]	0.316[f]	1.7[f]	497.34[f]	49.73[f]
Control	NA	NA	NA	NA	NA

NA: Not measured due to unobservable growth.

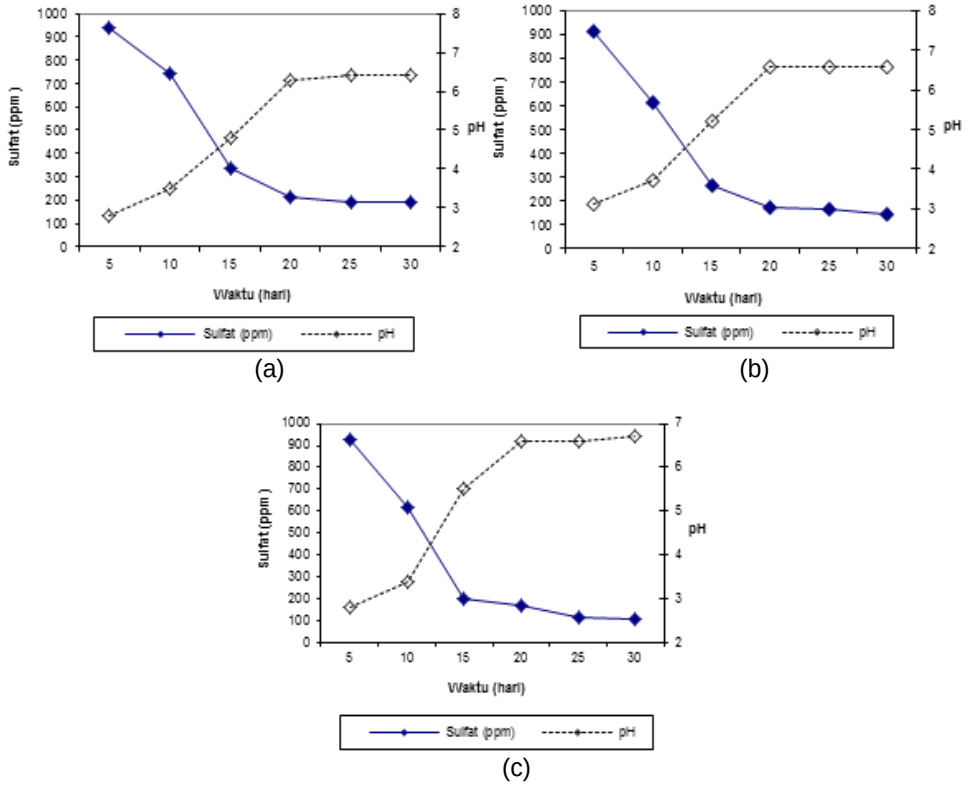

Figure 1. Graph showing the reduction of sulfate and increase in pH by (a) DPS 1703, (b) DPS 1705 and (c) DPS 1711 isolates.

and biochemical characterization were also performed. The results are shown in Table 5 and the photograph of the isolates in Figure 2. Endospores have very high resistance to heat and are not easily damaged by the effects of chemicals, dryness, radiation, acidity, and dormant for a very long time (Kato, 2016). In Figure 2c, the spherical endospores are in contrast to their rod-shaped vegetative cells. The color difference is caused by the absorption of green color of kit by endospora. Based on the observed characteristics shown by DPS 1703, DPS 1705 and DPS 1711 isolates.

The observed SRB-shaped group of SRB (Figure 2a), with a single arrangement is paired and aggregates. In Figure 2b, the aggregate bacterial cell appears from the DPS 1711 SRB isolate. Characteristics of the cells are Gram-negative, stem, motile and show only growth in anaerobic conditions.

In Figure 2, it is also clear that the morphological type of isolate observed are the form of a stem (bacillus) with a single or group arrangement. The pink bacterial cells showed Gram-negative cells that lose the complex of a purple primer in purple crystals when rinsing with alcohol

Table 5. Determination of morphological and physiological cell of Isolates.

Characteristic	Isolate		
	DPS 1703*	**DPS 1705***	**DPS 1711***
Gram test	Negative	Negative	Negative
Cell form	Rod	Rod	Rod
Colony form	Spherical-irregular	Spherical- irregular	Spherical-irregular
Colony colour	White gray	White gray	White gray
Motility	+	+	+
Oxsidase	+	+	+
Catalase	-	-	-
Anaerob	+	+	+
Aerob	-	-	-
Endospora	+	+	+
Carbon sources			
Lactate	+	+	+
Asetate	-	-	-
Phenol	-	-	-
Butirate	-	-	-
Formate	-	-	-
Growth temperature			
50 - 60°C	-	-	-
25 - 40°C	+	+	+
60 - 65°C	-	-	-
Other characteristics			
$H_2 + CO_2$	+	+	+
3-12% NaCl media	-	-	-
Gas formation during sporulation	-	-	-
Sulfide production	+	+	+

*Desulfotomaculum orientis.

(a) (b) (c)

Figure 2. (a) Rod form, (b) Aggregate cell, (c) spore spots are colored in contrast to their vegetative cells.

and stained with a counter-dye namely safranin. According to Dennis and Julia (2014), the differences in Gram-positive and Gram-negative cell wall structures cause different reactions in dyestuff permeability and the addition of the pale solution.

Most Gram-positive cell wall cells consist of peptidoglycan, while Gram-negative cell wall bacteria have high lipid content compared to Gram-positive cell wall cells. The lipids will dissolve in the pale solution, enlarge the pores of the cell wall and increase the solubility of the violet-iodine crystalline complex. On the other hand, Gram-positive bacteria produce compounds

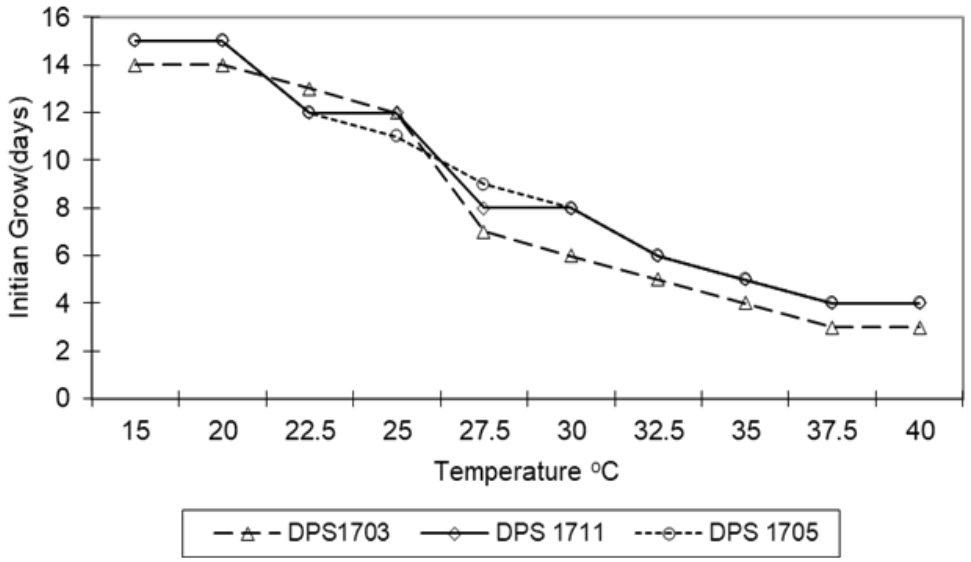

Figure 3. Comparison of temperature and growing time of SRB (Postgate B, pH 5).

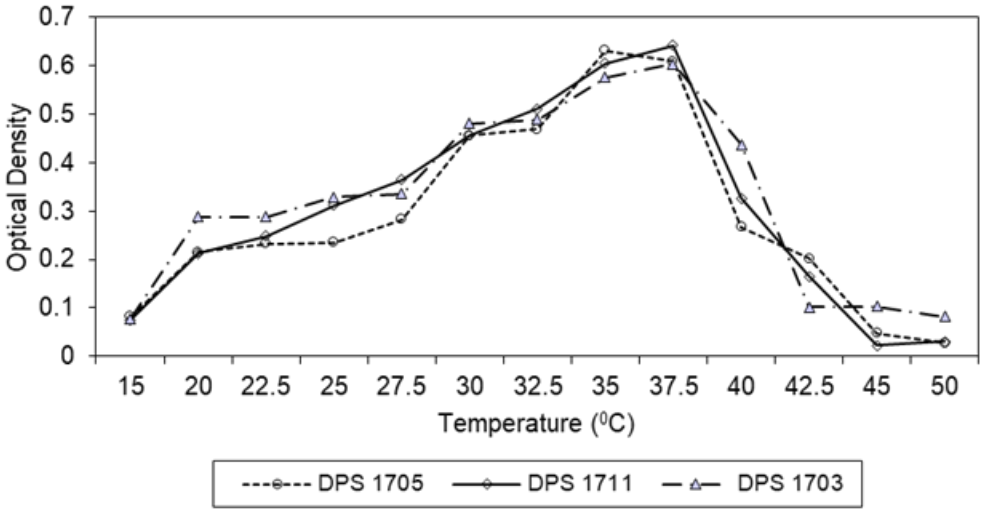

Figure 4. Effect of temperature on optical density of growth of DPS 1711, DPS 1705, and DPS 1703 (Postgate B, pH 5).

as crystalline violet-iodine ribonucleic acid.

Sulfate reducing bacteria (SRB) growth factors

The effect of temperature on SRB growth

Temperature is one of the important environmental factors that affect bacterial growth. Bacteria have specific temperature range of growth and optimal growth temperature. The minimum and maximum growth temperatures for microorganisms vary greatly, reflecting the range of temperatures in which they live (Païssé et al., 2013). Figure 3 shows the growth time range of DPS 1703, DPS 1705 and DPS 1711 isolates. At 20°C, SRB shows very slow growth, and a long acclimation time of 15 days is required. At room temperature, the growth of isolate was relatively rapid at 7 to 10 days, while at 45°C temperature the isolates did not grow. At the temperature range of 25 to 40°C, SRB Isolates showed the fastest growth especially DSS 1703 isolates. All isolates showed a similar growth time profile. The growth is also indicated by changes in the optical density of the media as a function of temperature (Figure 4). Figure 4 shows that

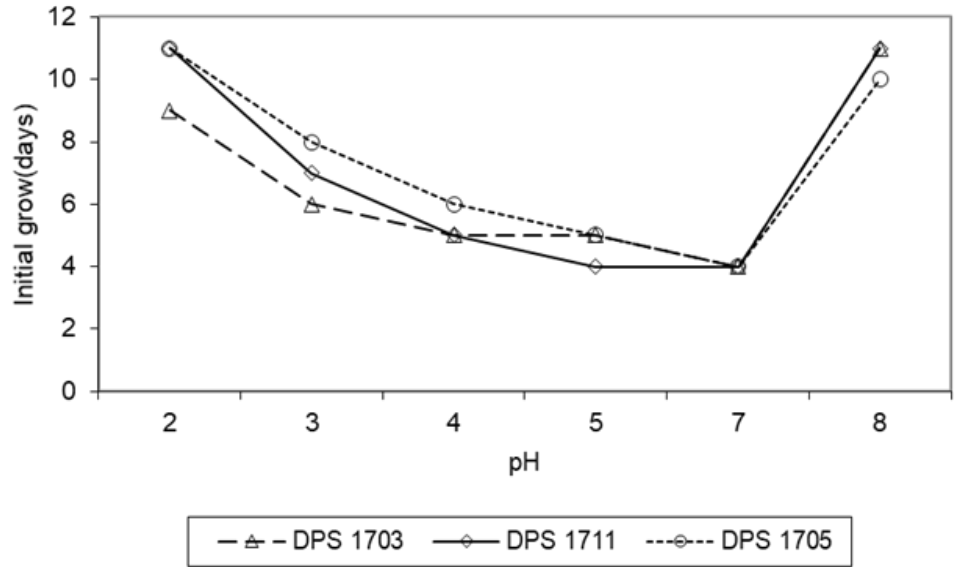

Figure 5. The curve of pH ratio and growing time (days) of DPS 1711, DPS 1705 and DPS 1703.

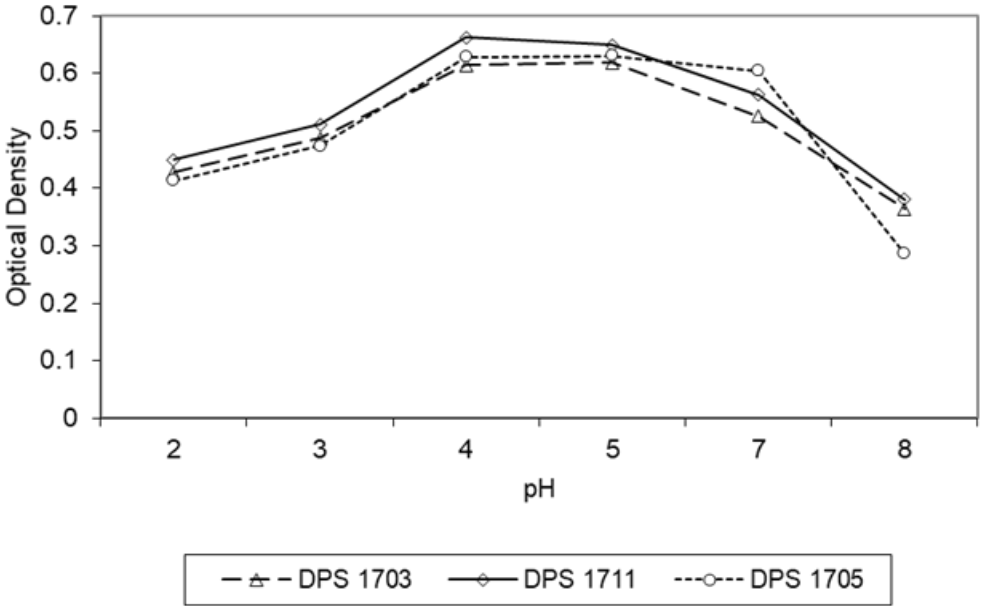

Figure 6. Effect of pH on optical density of growth of I DPS 1711, DPS 1705 and DPS 1703 (Postgate B, temperature 35°C).

the SRB isolates grow with optical density ranging between 0.30 and 0.65 at temperature range of 27 to 40°C with the best range of 35 to 37.5°C.

Effect of pH on growth of SRB

Microorganisms have a certain pH range for their growth, with optimum pH range of 5 to 9. Only certain species are able to grow under extreme conditions such as below pH 2 or above pH 10. Comparison of the activities of all SRB isolates at various pH, with a pH range of 2 to 9, is shown in Figure 5. At pH 2 and pH 8 the growth time of the three isolates was very slow and at pH 9 did not show growth at all. Optimal growth time occurs at pH 4 to 7 and fastest at pH 7. Figure 6 shows the optical density of DPS 1711, DPS 1705 and DPS 1703 isolates at various pH. Optimal optical density occurred at pH range of 4 to 5. The growth

Table 6. Sulfate reduction by DPS 1711, DPS 1705 and DPS 1703 at various initial sulfate concentrations.

[$SO_4^=$] Initial (ppm)	Sulfate reduction					
	DPS 1703		DPS 1705		DPS 1711	
	$SO_4^=$	Red (%)	$SO_4^=$	Red (%)	$SO_4^=$	Red (%)
500	53.07[a]	89[a]	21.54[b]	95[b]	13.48[c]	97[c]
1000	69.40[a]	93[a]	63.68[b]	94[a]	51.06[c]	95[bc]
2000	431.04[a]	78[a]	209.48[b]	89[b]	79.25[c]	96[c]
5000	1302.11[a]	74[a]	1071.50[b]	78[b]	922.54[c]	82[c]
8000	1704.32[a]	78[a]	1346.16[b]	82[b]	1309.41[c]	83[b]

of the isolates at low pH is the ability of SRB to interact with acidic environment to achieve optimal environment for its growth. Such interaction conditions and abilities vary for each microorganism. The time taken for SRB isolates to grow at lower pH is longer, which is in line with the length of time required to achieve optimum pH. Tolerance to low pH is only performed by extra cellular interaction, while intra-cellular activity requires a higher optimal pH condition.

Reducing sulfate of SRB

SRB has the ability to transfer electron or hydrogen to sulfate which acts as terminal electron acceptor. From the process of the redox reaction, the sulfate is reduced to sulfide. Sulfate reduction occurs in anaerobic conditions (Rückert, 2016). The main product of sulfate reduction depends on the substrate used. If the substrate used is hydrogen, then the product is hydrogen sulfide. When simple organic materials such as primary lactates are the electron donors, then the product is sulfide (Paulo et al., 2013). In Table 6 it was shown that the ability of the isolates to reduce sulfate for DPS 1703 (74 to 93%) is highest at the initial sulfate content of 1000 ppm, for DPS 1705 (78 to 95%) the highest is when the initial sulfate content is 500 ppm and for DPS 1711 (82 to 97%) the highest reduction percentage is under the initial sulfate content of 500 ppm. DPS 1711 has the highest sulfate reducing ability compared to the other isolates at all initial sulfate contents.

SRB uses sulfate (SO_4^{2-}), thiosulfate ($S_2O_3^{2-}$), sulfite (SO_3-) and other reducible sulfur ions as terminal electron acceptor in the respiration of its metabolism. In the presence of an organic compound or H_2 as an electron donor under anaerobic condition, the sulfate ion is reduced to sulfide following the equation (Torres-Alvarado et al., 2016):

$$2CH_2O + SO_4^{2-} \longrightarrow H_2S + 2HCO_3^-$$

$$5H_2 + SO_4^{2-} \longrightarrow H_2S + 4H_2O + 2e$$

Sulfate reduction can occur under wide range pH, pressure, temperature and salinity intervals. In a natural environment, sulfate can be a limiting factor for SRB activity, while simple organic compounds are available in the presence of other bacterial activity (Müller et al., 2014)

Conclusion

The selected bacteria namely (DPS 1711, DPS 1705 and DPS 1703) Sulfate reducing bacteria are identified as *D. orientis.* These bacteria exhibit wide range temperature and pH tolerance thus could be applied for treating highly polluted waste such as acid mine waste.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest

REFERENCES

Atlas RM (1993). Handbook of Microbial Media. CRC Press, Inc., 2000 Corporate Blvd., N.W., Boca Raton, Florida 33431. 556 p.

Barton LL, Faugue GD (2009). Biochemistry, physiology and biotechnology of sulfate-reducing bacteria. Advances in Applied Microbiology 68:41-98.

Dennis E, Julia G (2014). Corrosion of iron by sulfate-reducing bacteria: New Views of an Old Problem. Applied and Environmental Microbiology 80(4):1226-1236.

Kato S (2016). Micribial extracellular electron transfer and its relevance to iron corrosion. Microbial Biotechnology 9(2):141-148.

Müller AL, Kjeldsen KU, Rattei T, Pester M, Loy A (2014). Phylogenetic and environmental diversity of DsrAB-type dissimilatory (bi)sulfite reductases. International Society for Microbial Ecology Journal 9(5):1152-1165.

Païssé S, Ghiglione JF, Marty F, Abbas B, Gueuné H, Sanchez JM, Muyzer G, Quillet L (2013). Sulfate-reducing bacteria inhabiting natural corrosion deposits from marine steel structures. Applied Microbiology and Biotechnology 97:7493-7504.

Paulo RDM, Diogo R, Marcos ACB, Carlos MG, Angela B, Mariana VP, Patricia do RD, Vânia AV, Chapaval P (2013). Occurrence of sulfate reducing bacteria (SRB) associated with biocorrosion on metallic surfaces in a hydroelectric power station in Ibirama (SC)-Brazil. Brazilian Archives of Biology and Technology 56:5.

Rückert C (2016). Sulfate reduction in microorganisms—recent advances and biotechnological applications. Current Opinion in Microbiology 33:140-146.

Shiqiang C, Peng W, Dun Z (2014). Corrosion behavior of copper under

biofilm of sulfate-reducing bacteria. Corrosion Science 87(5):407-415.
Torres-Alvarado MR, Calva-Benítez LG, Álvarez-Hernández S, Trejo-Aguilar G(2016). Anaerobic microbiota: spatial-temporal changes in the sediment of a tropical coastal lagoon with ephemeral inlet in the Gulf of Mexico. Revista De Biologia Tropical 64(4):1759-1770.
Xu D, Li Y, Song F, Gu T (2013). Laboratory investigation of microbiologically influenced corrosion of C1018 carbon steel by nitrate reducing bacterium Bacillus licheniformis. Corrosion Science 77:385-390.