International Journal of Ecology and Environmental Sciences 44 (2): 191-198, 2018
ISSN: 2320-5199 (Online)

Occurrence and Activity of Sulphate Reducing Bacteria in Selected Estuarine Sediments of South Kerala, India

Salom Gnana Thanga Vincent [1]* and Rohini Raj [2]

Department of Environmental Sciences, University of Kerala, Kariavattom 695 581, Thiruvanathapuram, Kerala, India
Email: [1] salom@keralauniversity.ac.in; [2] rohinipushparaj@gmail.com
* Corresponding author

ABSTRACT

The occurrence of sulphate reducing bacteria in bottom sediments of selected estuarine ecosystems of South Kerala, and their activities were analyzed *in vitro*. The study covered three estuaries *viz*., Poonthura, Veli and Kadinamkulam of Thiruvananthapuram District. SRB were enumerated using the roll tube technique (modified Hungate method) in Postgate medium. Morphologically distinct colonies were isolated and transferred to Postgate broth in serum vials capped with butyl rubber stopper and aluminium cap assembly. The isolated cultures were used for further characterization and sulphate reduction studies. The viable count of SRB corresponded to organic carbon and sulphate concentration of the sediment. Molecular characterization by phylogeny of 16SrRNA genes revealed that strain S4 was *Citrobacter freundii* and S5 was *Bacillus tequilensis,* the activity of the latter as SRB is hitherto unreported. Nevertheless, both the identified cultures did not belong to the traditional group of SRB (δ proteobacteria). The growth of SRB and sulphate reduction was tested in two carbon sources (ethanol, acetate) and electron acceptors (sulphite, sulphate). When ethanol was used as substrate, both sulphate and sulphite as electron acceptors showed similar range of sulphide production. However, when acetate was used as substrate, sulphate as electron acceptor showed more sulphide production than sulphite. Acetate provided continued growth of *C. freundii* throughout the incubation period and sulphate reduction was more during decline phase of growth. This study indicates the presence of extremely diverse SRB communities that exhibit geographic patchiness and warrants more research.

Key Words: **S**ulphate Reducing Bacteria; *Citrobacter Freundii; Bacillus Tequilensis;* Sulphate Reduction; Hydrogen Sulphide

INTRODUCTION

Sulphate reducing bacteria (SRB) are anaerobic microorganisms that use sulphate as a terminal electron acceptor in the degradation of organic compounds. The ecological importance of SRB lies in their unique role in biogeochemical cycling of sulphur and carbon (Muyzer and Stams, 2008). Isotopic evidence indicates that sulphate reduction evolved approximately 3.7 Ga ago, well before the evolution of oxygenic photosynthesis and cyanobacteria (Baemgartner et al. 2006, Shen and Buck 2004). In environments rich in sulphate, sulphate reduction dominates mineralization which accounts for nearly 50% of organic matter decomposition in estuarine and coastal marine sediments (Jorgensen 1982). Vincent et al. (2017) reported sulphate reduction as the predominant terminal electron accepting process during organic matter degradation in Ashtamudi, a tropical estuary in South Kerala, India.

Dissimilatory sulphate reduction is the reduction of sulphate to sulphite to obtain energy for growth and maintenance, which is exclusively done by SRB (Castro et al. 2000). More than 33 species of SRB are known, most having been described in the last two decades (Sahrani et al. 2008). Molecular in-situ analysis based on 16SrRNA gene sequence suggest that many so far uncultivated sulphate reducing microorganisms inhabit various environments such as hot springs (Hugenholtz et

al. 1998); deep sea hydrothermal vent systems (Takai and Horikoshi 1999) old marine sediments (Ravenschlag et al. 2000) and subsurface aquifers (Fry et al. 1997). Rates of sulphur reduction depend on the available concentrations of electron donors and acceptors as well as on population densities of SRB in the sediments. The production of hydrogen sulphide often indicates the activity and presence of sulphate reducing micro-organisms in natural habitats and its presence is obvious by a characteristic smell and black precipitation of ferrous sulphide when iron minerals are present.

Apart from ecological importance, SRB also have considerable economic importance and hence, are a popular subject of scientific investigation. *Citrobacter freundii* was reported with a wide variety of industrial applications like biodegradation of tannic acid and decolorization of sulphonated azo dyes (Chen 2004). Although extensive studies regarding diversity and activity of SRB of varied ecosystems have been done throughout the globe, such attempts pertaining to tropical estuarine ecosystems are scanty. Many of the estuaries of South Kerala are polluted by various human activities and most of them are loaded with organic and inorganic compounds. Anaerobic condition conjoined with the production of hydrogen sulphide is a general phenomenon noticed in these estuaries. Hence, an attempt was made for the first time to isolate and characterize SRB from estuarine sediments of Thiruvananthapuram district, Kerala.

STUDY AREA

The study area includes three estuaries *viz*., Poonthura, Veli and Kadinamkulam of Thiruvananthapuram District, South Kerala, India **(**Figure 1).

Station 1, Poonthura estuary and the lower reaches of Karamana river (8° 25' - 8° 30' N latitude and 76° 55' - 77° 00' E longitude) receive enormous load of untreated domestic waste from Thiruvananthapuram city. Station 2, Veli lake (8° 31' - 8° 31' N latitude and 76° 52' 30"- 77° 53' 30" E longitude) is smallest brackish water lake in South West Kerala. In the close vicinity of the lake are two factories and a major tourist spot. Station 3 - Kadinamkulam is the largest lake (8° 35' - 8° 38' N latitude and 76° 52' E longitude). This estuary is the centre for fishing, aquaculture, tourism, sand mining and open dumping of waste. The striking feature of Kadinamkulam is the presence of coconut husk retting, which contributes to heavy load of organic pollution in the lake**.** Sediment samples were collected from polluted estuaries of Kadinamkulam, Veli, and Poonthura using grab samplers, brought to the laboratory in air tight bottles and refrigerated.

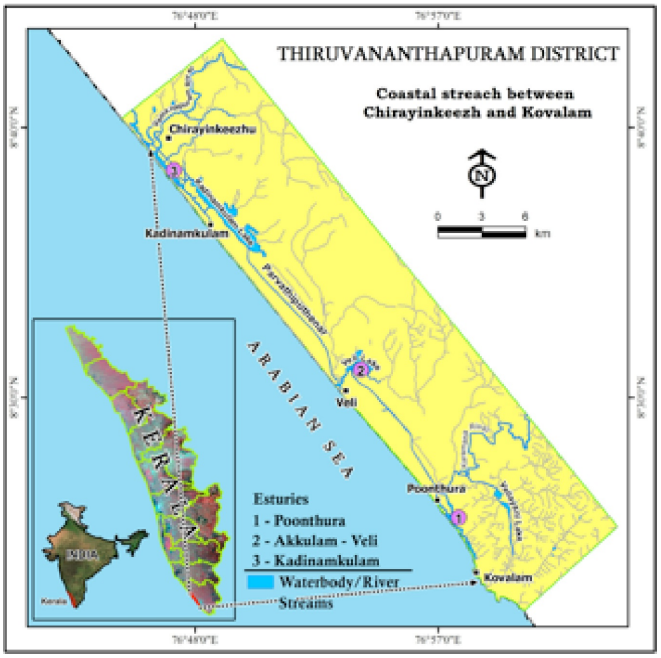

Figure 1. Study area indicating sampling sites

Enrichment, Enumeration and Isolation of SRB

The sediment sample was enriched in pre-reduced Postgate broth containing (g L^{-1} distilled water) KH_2PO_4 (0.5); Na_2SO_4/ Na_2SO_3 (1.0); NH_4Cl (1.0); $CaCl_2.2H_2O$ (0.01); $FeSO_4$ (0.1); yeast extract (1.0); ethanol (4.0 mL) or sodium acetate (1.0); ascorbic acid (0.1); agar (1.5); resazurin (0.1) (Postgate 1984). Resazurin was used as a redox sensitive dye to monitor the redox potential of the medium. This dye is dark blue in its inactive form (oxidized) and then turns pink (partially oxidized) and finally became colourless when the oxygen is removed. SRB was enumerated using the roll tube technique (modified Hungate method) (Ramasamy et al. 1992) in Postgate medium. Morphologically distinct colonies were isolated and transferred to Postgate broth in 60 mL serum vials capped with butyl rubber stopper and aluminium cap assembly. The isolated cultures were used for further characterization and sulphate reduction studies. The head space of vials and roll tubes were purged with high purity N_2 gas through a gassing manifold assembly to maintain anoxic conditions.

Assessment of Sulphate Reduction and Sulphide Production

All five bacterial isolates were tested for hydrogen sulphide production with two electron donors (ethanol or acetate) and electron acceptors (sulphate or sulphite). The experiments were performed in 60 mL serum vials capped with butyl rubber stopper and aluminium cap assembly containing mineral broth added with either of the electron donor and electron acceptor. Culture samples were taken during 24, 48 and 72 hours of incubation and analyzed for cell growth in terms of protein as well as for sulphate and sulphide concentrations.

Analytical Methods

The pH and EC of the estuarine sediments were analysed using pH meter and conductivity meter respectively. Sulphide was estimated by titrimetric method and sulphate by turbidometric method using spectrophotometer (Trivedi and Goel 1984). The organic carbon content of the sediment was analysed by wet oxidation method (Walkley and Black 1934).

Characterization and Identification of SRB

DNA was isolated from the liquid culture (strains S4 and S5), electrophoresed in 1% Agarose gel and visualized under UV. 16S r DNA was PCR amplified with F and R primers. Amplicon was electrophoresed in a 1% Agarose gel and visualized under UV. Concentration of the amplicon was checked in a Nanodrop ND 2000. The amplicon was purified using Nucleospin purification column (Macherey-Nagel) and sequenced with forward and reverse primers in ABI 3730xl cycle sequencer. Forward and reverse sequences were assembled and contig was generated after trimming the low quality bases. The sequence analysis was carried out using bioinformatic tool BLAST of NCBI. Based on maximum identity score, first few sequences were selected and aligned using multiple sequence alignment software ClustalW and dendrogram was constructed.

RESULTS

Selected characteristics of estuarine sediments are given in Table 1. The three sampling sites varied with reference to sediment characteristics. pH was highly acidic in Kadinamkulam (4.27) and slightly higher in Poonthura (5.42). In Veli, pH was near neutral (6.23). Electrical conductivity was maximum in Poonthura sediment (27.15 mS) followed by Kadinamkulam (13.53 mS), whereas, in Veli, it was very less (3.29 mS). The growth of SRB was observed as black colonies on the sides of the roll tubes, which, on further incubation formed a thick black growth throughout the tube (Figure 2). Population of SRB was found to be highest in Kadinamkulam (350 CFU g^{-1}), followed by Poonthura (200 CFU g^{-1}). Station 2 Veli showed considerably low alues (10 CFU g^{-1}).

Table 1. Characteristics of estuarine sediments at three sampling stations

Characteristics	Poonthura	Veli	Kadinamkulam
pH	5.42	6.23	4.27
EC (mS)	27.15	3.29	13.53
Total organic carbon (%)	5.05	1.84	11.71
Sulphate (mg g^{-1})	2.10	0.42	2.44
SRB (CFU g^{-1})	200	10	350

EC – Electrical Conductivity; SRB – Sulphate reducing bacteria; CFU – colony forming units

Figure 2. Growth of SRB in roll tube containing Postgate media

Five distinct colonies were isolated into serum vials containing Postgate broth for further purification and characterization. The bacterial strains S1, S2, S3 and S4 were gram negative cocci and S5 was gram positive rods. The strains S4 and S5 were characterized using 16S rRNA techniques; S4 was identified as *Citrobacter freundii* and S5 as *Bacillus tequilensis* based on nucleotide homology and phylogenetic analysis (Figure 3, 4a and 4b). The 16S rRNA gene sequence of the two cultures (S4 and S5) has been deposited in GenBank under the accession numbers KF053092 and KF298061 for *C. freundii* and *B. tequilensis* respectively.

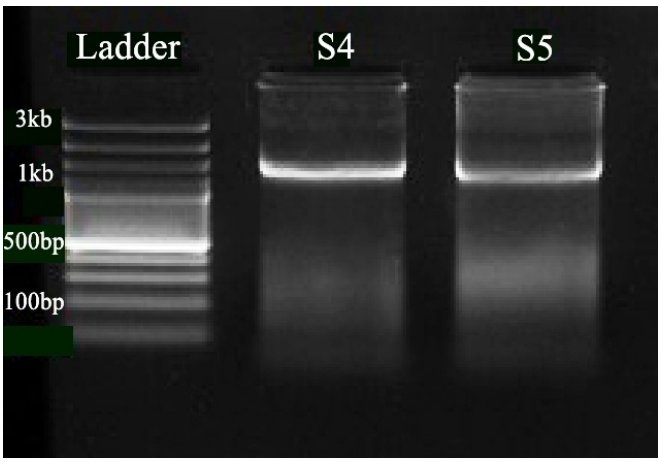

Figure 3. Amplicon photograph of S4 (*Citrobacter freundii*) and S5 (*Bacillus tequilensis*)

Five SRB isolates were tested for sulphide production *in vitro* using varied sources of electron donors and electron acceptors. When sulphate was used as electron acceptor and with both acetate as well as ethanol as electron donor, sulphide production and corresponding sulphate reduction was maximum by *C. freundii* (Figures 5 and 6). When acetate was used as electron donor, the growth of this bacterium was found to increase from the first to third day of incubation. However, with ethanol, growth of *C. freundii* was maximum at 24 hours after incubation, which decreased thereafter. However, the sulphate reduction increased with increasing incubation period. Nevertheless, all other SRB cultures showed increase in growth from 24 to 48 hours of incubation which decreased thereafter. For all SRB cultures, mean sulphide production was comparatively less when sulphite was used as electron acceptor with both acetate and ethanol as electron donors (Figures 7 and 8).

DISCUSSION

Acidic nature of water and sediments was reported to be the notable feature in coir retting zones of these estuaries. Depletion of oxygen and simultaneous production of hydrogen sulphide was the most conspicuous environmental damage in the retting zones (Nandan 1997). The sulphate content, SRB count and total organic carbon content showed similar trend in the

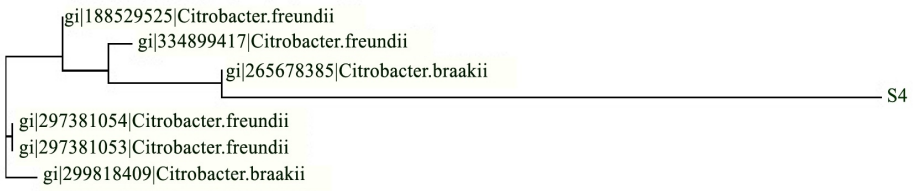

Figure 4.a. Dendrogram of S4 (*Citrobacter freundii*)

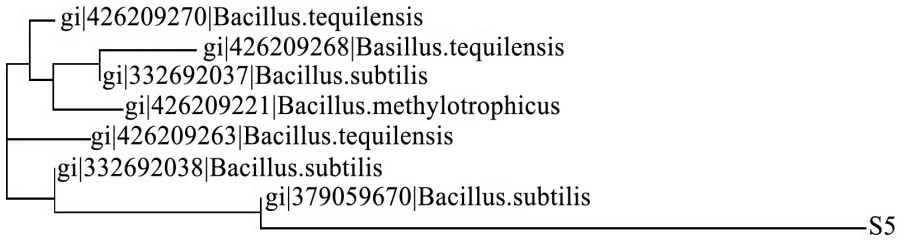

Figure 4.b. Dendrogram of S5 (*Bacillus tequilensis*)

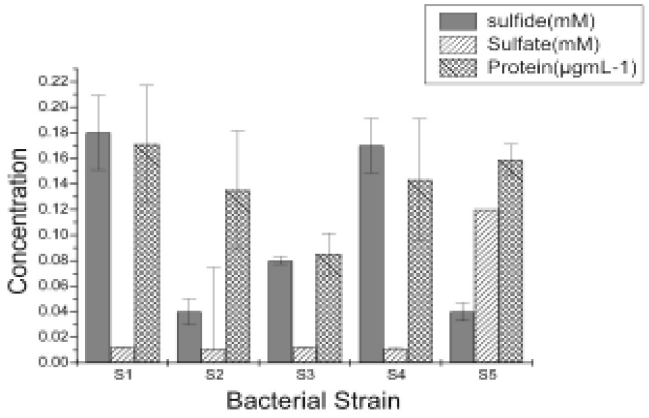

Figure 5. Sulphide (mM), sulphate (mM) and growth (µgmL^{-1} of cell protein) of SRB in media containing sulphate and acetate.

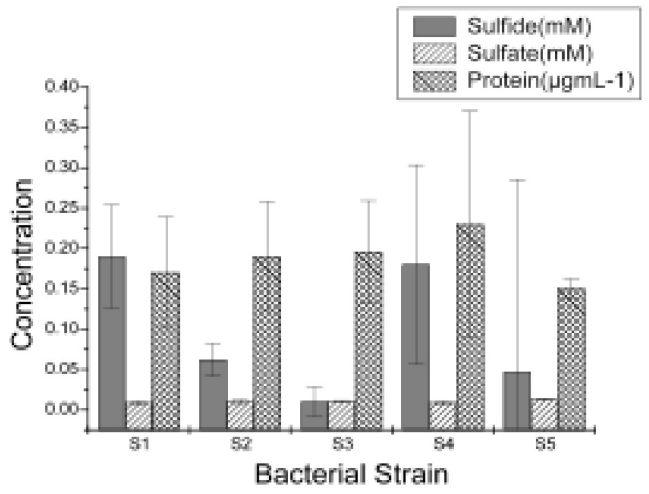

Figure 6. Sulphide (mM), sulphate (mM) and growth (µgmL^{-1} of cell protein) of SRB in media containing sulphate and ethanol.

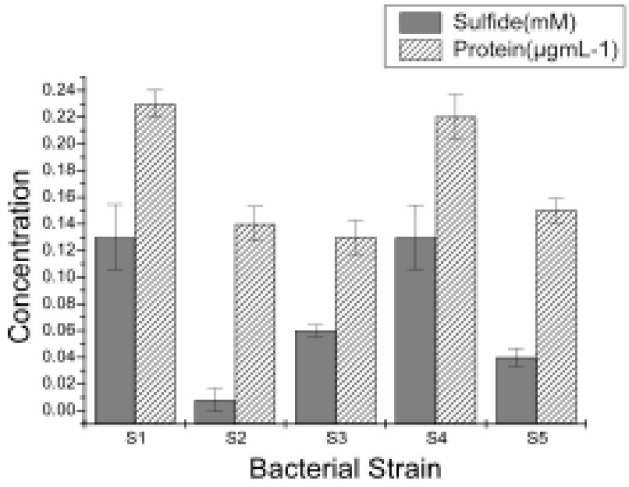

Figure 7. Sulphide (mM) and and growth (µgmL^{-1} of cell protein) of SRB in media containing sulphite and acetate.

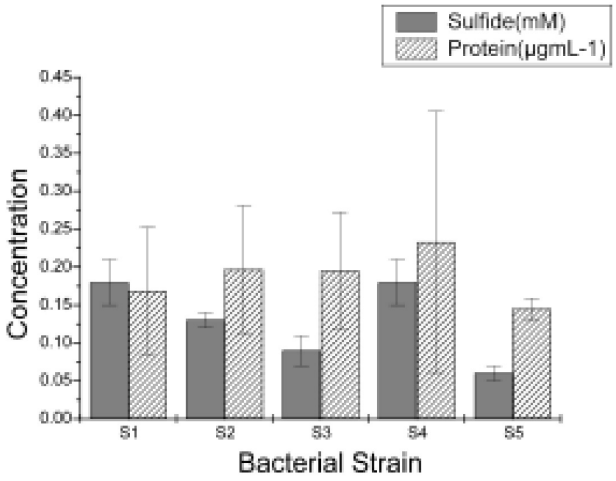

Figure 8. Sulphide (mM) and growth (µgmL^{-1} of cell protein) of SRB in media containing sulphite and ethanol.

sampling sites. The retting environment provides a competitive niche for specialized microbes and harbors a variety of microbes with high biodegradation potential (Reshma and Vincent 2011).

On enumeration, it was observed that station 3, which was the retting site in Kadinamkulam had the maximum number of bacterial colonies and corresponded to the high sulphate and organic carbon content, which may be attributed to retting activities in this site. The samples from station 2 which was taken from the less polluted sand bars of Veli estuary showed the least number of colonies (Table 1). This again corresponded to the lower concentration of organic carbon and sulphate in the sediment. This confirms the occurrence and preferential distribution of SRB in polluted sites having high sulphate and organic carbon content. The viable counts of SRB correspond to substrate concentration and availability of sulphate as electron acceptor (Parker et al. 1993). However, this is in contrast to the report by Perez-Jimenez and Kerkhof (2005) that SRB communities under chronic anthropogenic impact contain roughly half of the SRB population found in more pristine areas. A strong and distinct smell of hydrogen sulphide in certain parts of Ashtamudi estuarine sediment, Kerala suggested sulphate reduction as the prominent electron accepting process (Reshmi et al. 2014, Vincent et al.2017).

Interestingly, both the isolates do not belong to the traditional SRB group (Qui et al. 2009) i.e., the members of the delta class of the phylum proteobacteria (Perez-Jimenez and Kerkhof 2005). Nevertheless, the other three SRB cultures are yet to be characterized, which may reveal the presence of traditional SRB. *Citrobacter freundii* is a novel species and not much reports of its property of sulphate removal has been reported It has been used in the sulphate removal from tannery waste water (Zhao et al. 2011) and in the precipitation of copper as copper sulphide in acid mine drains (Qui et al. 2008). Higher SRB populations were reported even under most oxidized conditions (Bussmann and Reicchand 1991). Members of the order Bacillales are found in almost every environment and the unique feature is their great metabolic versality and ability to grow under physico-chemical extremes (Sass et al. 2008). One among the four general group of SRB is the gram-positive spore forming SRB, which is dominated by the genus *Desulfotomaculum*, placed within low GC gram-positive bacteria such as *Bacillus* and *Clostridium*. Currently, SRB are divided into four phylogenetic groups. Nevertheless, new divisions could be added as more information on the diversity of SRB in extreme environments becomes available (Castro et al. 2000). Wagner et al. (1998), using phylogenetic analyses of dissimilatory sulphite reductases, found sequences different from previously described sequences, which also suggests the possible presence of undescribed SRB. Anbar and Knoll (2000) analyzed the distribution of SRB communities in various geographical locations using dsr-TRFLP analysis and opined that a homogenous distribution *i.e.* everything is everywhere is not likely. This is true in the context that entirely different SRB were isolated from Kerala backwaters which are obviously unique tropical ecosystems. Nevertheless, microbial community diversity and structure is linked with nutrient parameters (Hafich et al. 2012)

When ethanol was used as substrate, both sulphate and sulphite as electron acceptors showed similar range of sulphide production. However, when acetate was used as substrate, sulphate as electron acceptor showed more sulphide production than sulfite. Acetate provided continued growth of *C. freundii* throughout the incubation period and sulphate reduction was more during decline phase of growth. The carbon source most frequently used by SRB can be divided into two major groups : simple compounds (methane, ethanol, acetic acid, sodium lactate, glucose, lactose) and complex organic materials (usually waste materials from the food industry, agriculture, forestry, excess sediments from wastewater purification plants) (Rzeczycka and Blaszez 2005).

Figures 9 and 10 show the pattern of sulphate reduction and sulphide production by *C. freundii* when grown in acetate or ethanol. Sulphate reduction was almost similar with both substrates and very negligible also. However, sulphide production was more pronounced and a steady increase was observed with ethanol compared to acetate. Mudyrk et al. (2000) reported a direct relationship between number of SRB and sulphide concentration; however, there was no such relationship with reference to sulphate concentrations. SRB inhabiting the bottom sediments were able to utilize three different organic substrates (lactate, propionate and acetate) as electric donors and and as carbon and energy sources.

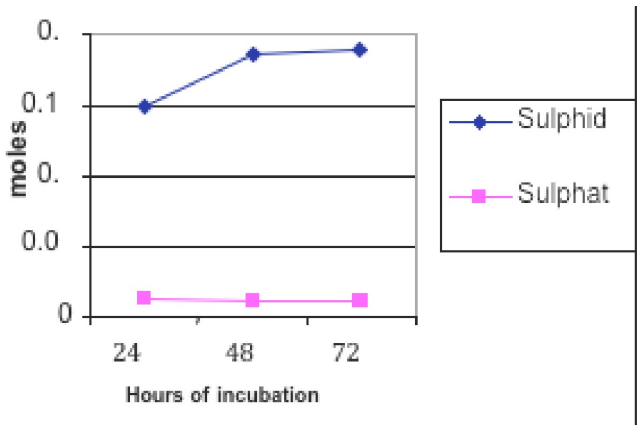

Figure 9 . Sulphate reduction and sulphide production by *C. freundii* with acetate

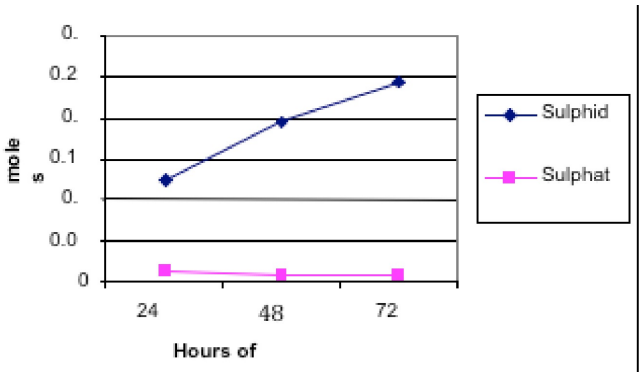

Figure 10. Sulphate reduction and sulphide production by *C. freundii* with ethanol

CONCLUSIONS

The present study revealed the occurrence of two unusual SRB in the tropical estuarine sediments of South Kerala. *C. freundii* and *B. tequilensis* belong to the non-traditional SRB and the latter as SRB is hitherto unreported. Further, the functional role of these organisms has to be studied in detail for further applications of SRB. *C. freundii* has been previously reported to be used in the sulphate removal from tannery waste water and in the precipitation of copper as copper sulphide in acid mine drains. Moreover, they are also efficient in decolorization of sulphonated azodyes. Hence, SRB being important regulators of a variety of processes including ecosystem functioning and environmental remediation warrants further investigations on bioprospecting novel SRB from similar ecosystems.

ACKNOWLEDGMENTS

We acknowledge the Department of Environment and Climate Change, (DoECC), Government of Kerala, India, for funding this research work. The help of Dr Baijulal Bhaskar and Dr Jissy Jyothi in preparation of study area map is also gratefully acknowledged.

Author contributions: The first author designed the experiments, analysed the data and prepared the draft manuscript. The second author did the experiments and edited the manuscript.

REFERENCES

Anbar,A.D. and Knoll,A.H. 2002. Proterozoic ocean chemistry and ecolution: a bioorganic bridge? Science 297: 1137-1142.

Baemgartner, L.K.; Reid, R.P.; Uprez, C.D.; Decho, A.W.; Buckley, J.R.; Spear, D.H.; Przekpy, K.M. and Visschor, P.T. 2006. Sulfate reducing bacteria in microbial mats – changing paradigms, new dimensions. Sedimentary Geology 185: 131-145.

Bussman, I. and Reicchand, W. 1991. Sulfate reducing bacteria in temporarily oxic sediments with bivalves. Marine Ecology Progress Series 78: 97-102

Castro, H.F.; Williams, N.H. and Ogram, A. 2000. Phylogeny of sulfate-reducing bacteria. FEMS Microbiology Ecology 31 :1-9.

Chan, G.F. 2004. Studies on the Decolourization Mechanism of Azo Dyes by *Citrobacter freundii* A1 from the Molecular and Enzymatic Aspects. Ph.D Thesis, Universiti Teknologi Malaysia, Johor.

Fry, N.K.; Fredrickson, J.K.; Fishban, S.; Wagner, M. and Stahl, D.A. 1997. Population structure of microbial communities associated with two deep anaerobic alkaline aquifers. Applied and Environmental Microbiology 63: 1498-1504.

Harich, K.; Perkins, E.J.; Hauge, J.B.; Barry, D. and Eaton, W.D. 2012. Implications of land management on soil microbial communities and nutrient cycle dynamics in the lowland tropical forest of northern Costa Rica. Tropical Ecology 53: 215 – 224.

Hugenholtz, P.; Pitulle, C.; Hershberger, K.L. and Pace, N.R. 1998. Novel division level bacterial diversity in Yellowstone hot spring. Journal of Bacteriology 49: 1157-1163.

Jorgensen, B.B. 1982. The role of sulphate reduction in the mineralization of organic matter. Nature 296: 643-645

Mudryk, Z.J.; Podgorska, B. and Bolalek, J. 2000. The occurrence and activity of sulphate-reducing bacteria in the bottom sediments of the Gulf of Gdansk. Oceanology 42: 105-117

Muyzer, G. and Stams, A.J.M. 2008. The ecology and biotechnology of sulphate-reducing bacteria. Nature Reviews 6: 441-445.

Nandan, S.B. 1997. Retting of coconut husk – a unique case of water pollution in the South West Coast of India. International Journal of Environmental Studies 52: 335-355.

Parker, R.J.; Dowling, N.J.E.; White, D.C.; Herbert, R.A. and Gibson, G.R. 1993. Charactertization of sulphate reducing bacterial population within marine and estuarine sediments and different rates of sulphate reduction. FEMS Microbiology Ecology 102: 235-250.

Perez-Jimenez, J.R. and Kerkhof, L.J. 2005. Phylogeography of sulfate-reducing bacteria among disturbed sediments, disclosed by analysis of the dissimilatory sulphite reductase genes (dsrAB). Applied and Environmental Microbiology 71: 1004-1011.

Postgate, J.R. 1984. The Sulphate Reducing Bacteria. Cambridge University Press, Cambridge. 151 pages.

Qiu, R.; Zhao, B. and Jinling, L. 2009. Sulphate reduction and copper precipitation by a *Citrobacter* sp. isolated from a mining area. Journal of Hazardous Materials 164: 1310-1315.

Ramasamy, K.; Kalaichelvan, G. and Nagamani, B. 1992. Working with Anaerobes: Methanogens-a Laboratory Manual. Fermentation Laboratory, Tamil Nadu Agricultural University, Coimbatore, India. 87 pages.

Ravenschlag, K.; Sahm, K.; Jorgensen, B.B. and Amann, R. 2000. Community structure, cellular rRNA content, and activity of sulphate reducing bacteria in marine Arctic sediments. Applied and Environmental Microbiology 66: 3592-3602.

Reshma, J.K. and Vincent, S.G.T. 2011. Quantification of polyphenols during retting and characterization of bacteria from the Kadinamkulam backwaters, Kerala. Journal of Environmental Biology 32: 133-135.

Reshmi.R.R.; Nair, K.D.; Zachariah, E.J. and Vincent, S.G.T 2014. Methanogenesis: Seasonal changes in human impacted regions of Ashtamudi estuary (Kerala, South India). Estuarine Coastal and Shelf Science 156: 144-154. .

Rzeczycka, M. and Blaszezyk, M. 2005. Growth and activity of SRB in media containing phosphate gypsum and different sources of carbon. Polish Journal of Environmental Studies 14: 891-895

Sahrani, F.K.; Ibrahim, Z.; Yahya, A. and Aziz, M. 2008. Isolation and identification of marine sulphate-reducing bacteria

Desulfovibrio sp. and *Citrobacter freundii* from Padir Gudang, Malaysia. Sains Malaysiana 37: 365-371.

Sass, A.M.; McKew, B.A.; Sass, H.; Fitchell, J.; Timmis, K.N. and McGenily, T.J. 2008. Diversity of Bacillus like organisms isolated from deep sea hypersaline anoxic sediments. Saline Systems 4**:** 8.

Shen, Y. and Buck, R. 2004. The antiquity of microbial sulfate reduction. Earth Science Reviews 64: 243-212.

Takai, K. and Horikoshi, K. 1999. Genetic diversity of archae in deep sea hydrothermal vent environments. Genetics 152: 1285-1297.

Trivedi, R.K. and Goel, P.K. (Editors). 1984. Chemical and Biological Methods for Water Pollution Studies. Environmental Publication, Karad. 215 pages.

Vincent, S.G.T.; Reshmi, R.R.; SalahudeenJ.H.; Deepa Nair, K. and Varma, A.K. 2017. Predominant terminal electron accepting processes during organic matter degradation : spatio-temporal changes in Ashtamudi estuary, Kerala, India. Estuarine Coastal and Shelf Science, 198: 508-517.

Wagner, M.A.; Roger, J.; Fax, J.L.; Brusseau, G.A. and Stahl, D.A. 1998. Phylogeny of dissimilatory sulphite reductases supports an early origin of sulphite respiration. Journal of Bacteriology 180: 2975-2982.

Walkley, A and Black, I.A. 1934. An examination of the Degtjareff method for determining organic carbon in soils: Effect of variations in digestion conditions and of inorganic soil constituent. Soil Science 63: 251-263.

Zhao, C.; Yang, Q.; Chen, W.; Li, H. and Zhang, H. 2011. Isolation of a sulphate reducing bacterium and its application in sulphate removal from tannery waste water. African Journal of Biotechnology 10**:** 11966-11971.

Received 9 November 2017
Accepted 23 March 2018