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# CULTIVATION, ISOLATION AND IDENTIFICATION OF SULFATE REDUCING BACTERIA EMPLOYING HUNGATE TECHNIQUE

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**Abstract:** Obligate anaerobes are the most complicated establishment in the nature, which survive only in absence of molecular oxygen. The major challenge to cultivate anaerobes is lack of well-equipped anaerobic facility in the laboratory. Hungate roll tube technique is generally used for anaerobic isolation, wherein roll tubes are employed for isolation of bacterial colonies. In the present study the roll tubes were replaced with serum bottles to cultivate and isolate sulfate reducing bacteria as these are more convenient and reliable. A total 5 isolates were recovered from open drains of Tung Dhab region of Amritsar district (Punjab), India based on colony morphology which were further screened on the basis of gram staining, and 16S rRNA analysis. Sulfate reducing bacteria (BN-15) was finally identified as *Clostridium indolis* BN-15. Substrate *i.e.* short chain volatile fatty acids and NaCl utilization patterns were also observed for finally screened isolate.
**Keywords:** Anaerobic isolation; *Clostridium* species; Hungate roll tube; Sulfate reducing bacteria.
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## INTRODUCTION

Sulfur exists in the form of sulfate in sediments or pyrite/gypsum in rocks, where following the reduction/oxidation of sulfate/sulfide, nourish many micro-flora and fauna. Sulfur is a major constituent of the biogeochemical sulfur cycle as sulfur has a broad range of oxidation states, from -2 to +6. It can be transformed both chemically and biologically to various sulfur intermediates. Sulfate is consumed by sulfate reducing bacteria (SRB) and companions of archaea/bacteria, which uses it as a terminal electron acceptor for its reduction into sulfide when combined with enzymes and amino acids. Biochemical reactions representative of oxidation (chemolithotrophic sulfate reducers) and reduction process (dissimilatory sulfate reducers) generates metabolic energy. Sulfate reducing bacteria are widely distributed in nature, dominated mainly in extreme environments like marine sediments estuarine, saltmarsh sediments, environment of landfills, freshwater lakes, activated sludge systems, and acidic sediments representing its obligate anaerobic nature (Pimenov *et al*. 2014; Xia *et al*. 2014; Andrea *et al*. 2015; Brand *et al*. 2015; Colin *et al*. 2017; Cui *et al*. 2017; Yang *et al*.2017; Kharrat *et al*. 2017). The SRB are effectively adapted to almost all the ecosystem of the planet, where stringent anaerobic conditions prevail. In these ecosystems, they have to face severe physicochemical conditions. The SRB can be gathered into seven phylogenetic ancestries, two among the archaea and five among the bacteria which are constructed based on the comparative study of 16S rRNA sequences (Muller *et al*. 2014). Depending upon the substrate requirement sulfate reducers can be categorized into two groups: (a) Organotrophs- a microbial community which uses carbon complexes, such as lactate, acetate, and pyruvate as electron donors for *e.g.* bacterial community

prevailing in microbial mats (Gallagher *et al*. 2012). (b) Lithotrophs– a microbial community which consumes hydrogen as an electron donor resulting in iron corrosion (Enning and Garrelfs, 2014). Massive range of unusual sulfate reducers have been well-defined over the previous years that have the capability to grow on different substrates, including carbon monoxide, amino acids and one-carbon compounds, such as formate and methanethiol (Hansen and Blackburn, 1995; Beller *et al*. 1996; Baena *et al*. 1999; Tanimotto and Bak, 2014; Parshina *et al*. 2015). Electron acceptors which are reduced by the prokaryotes in anoxic ecosystem include nitrate, manganese (IV), $CO_2$, ferric iron, elemental sulfur, sulfate, protons and some of the less available form of natural elements (Rabus *et al*. 2013). The SRB can also consume thiosulfate, sulfite, and sulfur which consequently yield sulfate and sulfide (Leavitt *et al*. 2014). Besides benzoate and phenol, aromatic hydrocarbons i.e. toluene and ethyl benzene are also degraded with sulfate reduction by a number of SRB (He *et al*. 2013; Kuppardt *et al*. 2014). More than 50% of the organic carbon mineralization has been reported by the process of sulfate reduction in many marine environments, which showed the prominence of sulfate reducers in both the sulfur and carbon cycles, revealing why SRB have been studied much so far (Rabus *et al*. 2003). For isolation of anaerobic bacteria, several modifications have been made to the original technique described by Hungate (Bryant, 1972; Miller and Wolin, 1973). Present study was aimed to isolate anaerobic bacteria using Hungate technique by employing serum bottles which provides reliability to this technique.

## EXPERIMENTAL

**Sample collection:** The open drains of Tung Dhab region of Amritsar district (Punjab), India were chosen as sampling site because of seasonal water streams turn out to be a major source of pollution. These water streams get polluted when merged to the drains, which also received sewage water and industrial sludge (Kaur and Anish, 2016). Sludge sample was collected from contaminated site (Figure 1) at the depth of 30 cm using a long pipe and quickly transferred in anaerobic diluents by aseptic syringe.

**Figure 1. Collection of sludge sample from contaminated site**

**Anaerobic Media Preparation:** Howard and Hungate medium (Howard and Hungate, 1976) was used for the isolation of sulfate reducing bacteria from sludge sample with slight modifications. Serum bottles of (100 mL) were used for sampling, diluents, media preparation and for cultivation. All arrangement of anaerobic media was handled under oxygen free $CO_2$ atmosphere using standard anaerobic procedures as per the culture techniques described by Hungate, (1969). The anaerobic broth was prepared with 3 crucial steps *i.e.* (a) initial boiling of media, (b) flushing of $CO_2$ into the media by engaging gassing manifold system in which $H_2$ and $CO_2$ were circulated over copper fillings at 350°C to eliminate the remains of oxygen (c) finally added reducing solution containing L- cysteine hydrochloride. Solution-A ($Na_2SO_4$, 5.0g/L; NaCl, 2.0g/L; $(NH_4)_2SO_4$, 3.0g/L; $KH_2PO_4$, 3.0g/L; $CaCl_2{\cdot}2H_2O$, 0.6g/L; $MgSO_4{\cdot}7H_2O$ 0.6g/L) and solution-B ($K_2HPO_4$, 3.0g/L) were mixed in equal amount (17 mL/100mL). Yeast extract (1g/L), tryptone (1g/L), soluble carbohydrates (1g/L), resazurin (1g/L) thioglycolic acid (0.14mL/L), ascorbic acid (100mg/L) and rest of distilled water was added and boiled at 50-60°C until the medium color changed from colorless to light pink. The medium was cooled immediately in ice water bath and $CO_2$ was flushed into the medium by adding $NaHCO_3$ (5g/L) and L-cysteine hydrochloride (0.05g/L) in the final step. Similar steps were performed to

prepare agar medium by addition of 1.5% agar to the previous broth medium. The colorless or slight yellowish appearance of medium (broth/agar) was an indication of perfect anaerobic conditions, while any pinkish coloration indicates aerobic condition (Joblin, 1999). Medium (20 mL) was poured in each serum bottles and autoclaved at 121°C for 15 min. Sodium formate (0.5% w/v), was added to the pre reduced medium before autoclaving and treated with the gas mixture (<200 kPa) of $H_2$:$CO_2$ (4:1). 300µL of 10% $FeSO_4.7H_2O$ was added in anaerobic molten media maintained at 50°C with the help of sterile syringeas it resulted in the development of black color colonies. The diluted sludge sample was added to molten media and rolled over the ice bucket in such a way that media should spread evenly inside serum bottles. The serum bottles were incubated at 39°C for 2-3 days and observed for development of black color colonies. Bacterial colonies were carefully picked using sterile hypodermic needle and transferred to serum bottles carrying anaerobic broth. Serum bottles were closed with help of butyl rubbers and sealed with aluminum caps with the help of sealer. Glycerol stock was prepared in the same way as that of anaerobic diluent, where 20% of glycerol was used to maintain the anaerobic cultures at -80°C.

**Morphological characterization of isolates:** Isolated microorganisms were identified based on their colony morphology and subjecting them to microscopic examination by standard gram staining procedure.

**Molecular identification of sulfate reducing bacteria:** Genomic DNA of isolates was extracted by the method described by Pitcher *et al*., (1989). Anaerobic broth carrying 1 mL of pure cultures was centrifuged at 17,000 X g for 5 min at 20°C. The pellet was washed with high grade sterile water and processed for isolation of genomic DNA. The DNA samples were subjected to PCR amplification of 16S rRNA gene using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The amplification was carried out in a 100 µL volume containing, 4 unit Taq polymerase (Bangalore Genei, Bangalore, India), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.4 µM of each primer and 8 µL of template DNA in 1X PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 8.3). The reaction mixtures were exposed to PCR thermal cycler (C1000 Touch™ Thermal Cycler - Bio-Rad), with temperature conditions as follow. 94°C for 4 min (30 cycles), 94°C for 40 s, 55°C for 1 min, 72°C for 1.5 min and a final extension step at 72°C for 10 min. The PCR amplified DNA were purified with a GeneGET purification kit (Thermo Fisher Scientific). The PCR products of partial 16S rRNA gene were sequenced by (Xcelris Genomic Centre, Ahmedabad, India). The 16S rRNA gene sequences were analyzed using Chromas 2.5.1 and aligned to those of closely related bacterial species available at GenBank database using BLASTn program. Bacterial isolates were identified based on the percentage of sequence similarity (C97%) with that of a prototype strain sequence in the GenBank.

**Substrate Utilization:** The metabolic characterization of isolate was conducted by addition of different substrate with the help of sterilized filter (0.2 µm pore size) to anaerobic Howard and Hungate medium. The growth of isolate capable of using different substrate as electron acceptor was measured using basal medium supplemented with short chain volatile fatty acids i.e. acetate (20 mM), butyrate (20 mM), propionate (20 mM), isovalerate (20 mM). All serum bottles were secured with butyl rubber containing anaerobic medium. Substrate utilization was performed by measuring cell density at 665 nm after 24 h incubation on a spectrophotometer. The experiment was set in triplicates, where inoculated serum bottle served as negative control.

**NaCl Tolerance:** The anaerobically prepared medium was supplemented with different concentration of NaCl (0.5, 1, and 1.5% w/v) to check the salt tolerance of SRB isolate. The serum bottles were inoculated with culture grown at 39°C for 24h. The optical density was recorded at 665 nm using spectrophotometer. Similarly, the experiment was set in triplicates where un-inoculated serum bottle acted as negative control.

## RESULTS AND DISCUSSION

### Morphological characterization of isolates

1mL of diluted inoculum was inoculated in the molten agar containing serum bottles, employing Hungate roll tube method (Figure 2a, c). After 24 h incubation, colonies were differentiated on the basis of color, shape, size, elevation, margin, and structure. Total 5 isolates recovered, showing black colored colonies, round, oval and convex type morphology selected from sample of contaminated site (Figure 2b). Microscopic examination of gram staining slides revealed purple (gram +ve); rods with bulging end (Figure 2d). Mesophilic, non-sporing, Gram's negative sulfate reducing bacteria are mainly of *Desulfovibrio* genus which are abundantly prevalent in nature, oxidizing organic compounds partially, while gram positive bacteria of *Desulfotomaculum* and *Clostridium* genera are commonly sporulated (terminal spores) with complete and incomplete oxidizing species (Fauque, 1995).

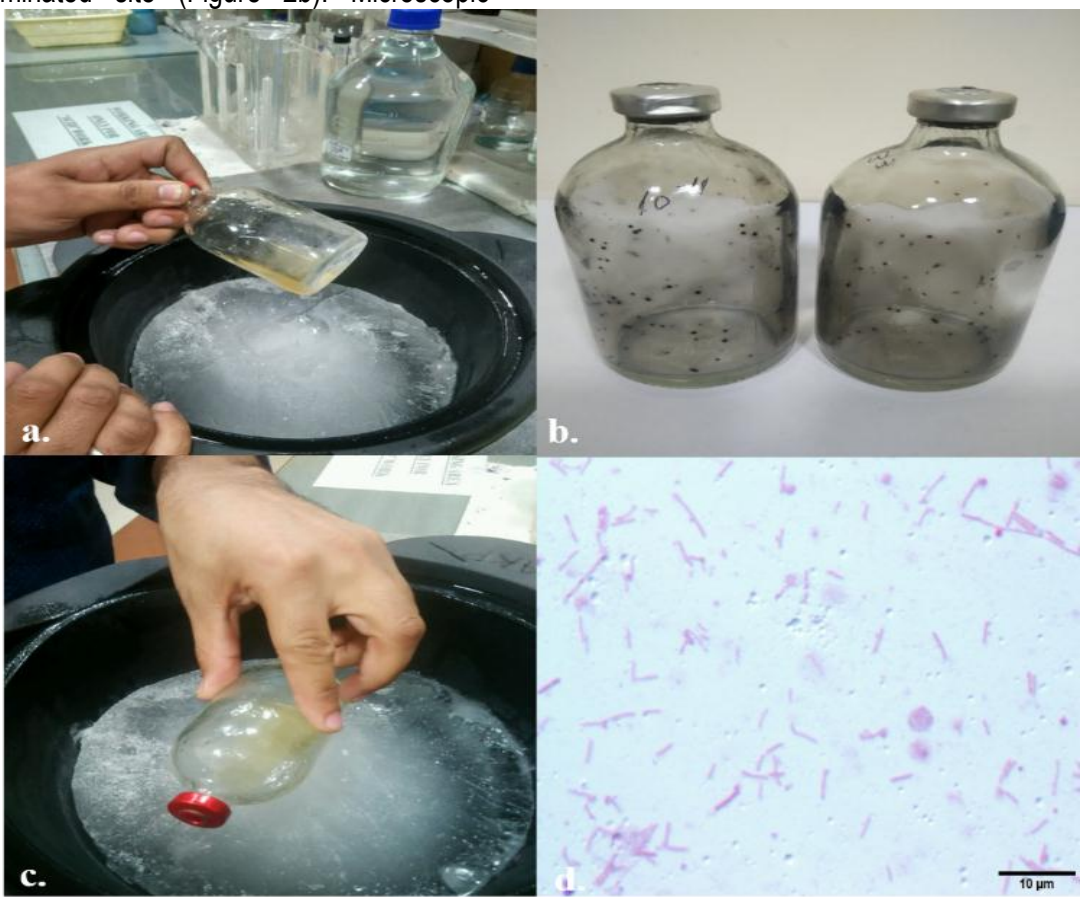


**Figure 2. Morphological Characterization:** (a,c) Molten Agar Media in Serum Bottles rolled over ice for even distribution; (b) Black color colonies of Sulfate reducing bacteria; (d) Microscopic view of gram reaction of selected isolate (*Clostridium indolis* BN-15)

### Molecular Identification

Primary identification of bacteria was done on the basis of colony morphology and microscopic examinations. Since dark black color bacterial colonies represents the sulfate reducing bacteria. The black color was result of precipitation of insoluble metal sulfide when react with hydrogen sulfide. Although cysteine-decomposing bacteria can cause blackening of colonies that may give false positive results or media composed of ascorbic acid as sole reducing agent are not always reliable (Postgate, 1963). Therefore these isolates were further confirmed by 16S rRNA analysis. The BLAST outcome of 5 isolates (Figure 3) revealed that four isolates *viz.,* 1, 2, 3 and 4 exhibit high resemblances to the prototype strain of *Escherichia coli*, where 5$^{th}$ isolate was identified as *Clostridium indolis*. *Escherichia coli* generally belong to *Gamma-proteobacteria*

class of *Proteobacteria* phylum. Majority of sulfate reducing bacteria basically belongs to *Delta-proteobacteria* class of *Proteobacteria* phylum and *Firmicute* phylum, where *Clostridium indolis* belong to *Firmicute* phylum. Sequence data of 5th isolate *i.e.* BN-15 was finally screened and deposited in NCBI gene bank library (Accession No: MF149975).

**Substrate Utilization**

Isolate BN-15 designated as *Clostridium indolis* was enriched with different substrates *i.e.* acetate, propionate, butyrate, and isovalerate. Positive growth response showed the selected isolate *Clostridium indolis* BN-15 towards acetate, propionate and butyrate. However no growth was observed in isovalerate (Figure 4) indicative of its non-utilization. During anaerobic degradation, complex organic compounds get converted to simpler one to release acetate, propionate, and butyrate, cooperatively stated as volatile fatty acids (VFA), and considered as significant electron donors for sulfate-reducing bacteria (Winfrey and Ward, 1983). Diagenetic environments with temperatures underneath 85°C composed formate, acetate, propionate, and butyrate as key intermediates in the anaerobic degradation of the organic matter (Magot, 2005).The process of anaerobic degradation became more complex in presence of sulfate reducers, which competes with the methanogens for common substrate (Baea *et al*. 2015).

**NaCl Tolerance**

Salt tolerance of selected isolate *Clostridium indolis* BN-15 was carried out by observing its growth at varied NaCl concentrations. Positive growth response was observed at 0.5 and 1% of NaCl concentrations, whereas the growth was inhibited at 1.5% of NaCl concentration (Figure 5.). Most bacteria yield high intracellular concentration of organic osmotic solutes at high energetic cost. Sulfate reducing bacteria tolerating 5% NaCl or more are well known which is not surprising at all (Zobell and Rittenbergs, 1948). Extreme environments including soda lakes contain high alkaline and saline conditions. Complex mechanism and adaptation of cytoplasmic membrane make them adaptive in such conditions. Significant sulfate reducing rates (between 12 and 423 µmol/dm$^3$ per day) were observed for most lakes, even at a salinity of 475 g/L (Foti *et al*. 2007).

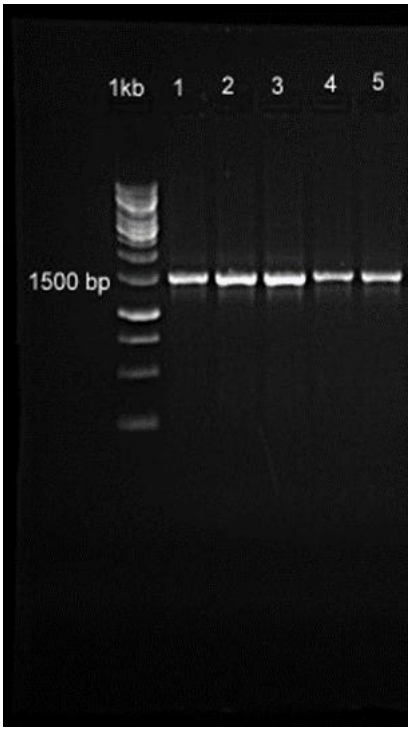


**Figure 3. Representative Electro-phoretogram of amplified 16S rRNA enconding region of 5 isolates as compared with GeneRuler 1kb DNA Ladder**

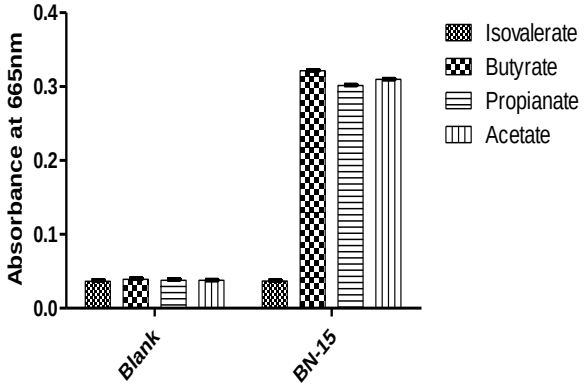


**Figure 4. Substrate Utilization Pattern of selected *Clostridium indolis* BN-15; Blank-Negative Control**

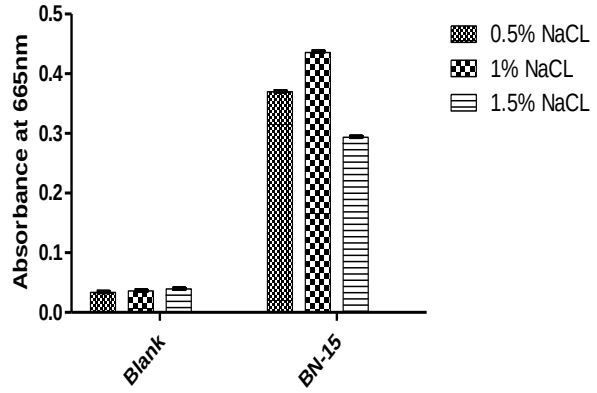


**Figure 5. Salt tolerance of selected *Clostridium indolis* BN-15; Blank- Negative Control**

## CONCLUSION

Till now, very few methods for culturing of anaerobic bacteria are available *e.g.* anaerobic gas pack jar and anaerobic chamber. Another robust technique to cultivate and isolate anaerobes is Hungate roll tube technique, which is highly efficient. Instead of Hungate tubes we used serum bottle as they can provide more surface area for growth of bacteria and incubated colonies can be picked conveniently. Moreover it becomes an easy task to visualize and differentiate colonies on the basis of their morphology. Although the preparation of pre-reduced media is little time-consuming. It was not our purpose to compare the already established methods for cultivation of anaerobic bacteria. An outline was derived from current research that isolation and cultivation of anaerobic bacteria can be feasible in the laboratory where well-equipped facilities are lacking especially the anaerobic chamber. Thus, serum bottles provide flexibility, credibility and reliability to the existing technique which provide an easy access to deal with the isolation of anaerobes.

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## REFERENCES

Andrea I.S., Stams A.J.M., Hedrich S., Nancucheo I., Johnson D.B. (2015). *Desulfosporosinus acididurans* sp. nov, an acidophilic sulfate-reducing bacterium isolated from acidic sediments. *Extremophiles,* 19(1):39-47.

Baea H.S., Holmesb M.E., Chantonb J.P., Reddya K.R., Ograma A (2015). Distribution, activities, and interactions of methanogens and sulfate-reducing prokaryotes in the Florida Everglades. *Appl. Environ. Microbiol.,* 81(21):7431-7442.

Baena S., Fardeau M.L., Ollivier B., Labat M., Thomas P., Garcia J.L., Patel B.K.C (1999). *Aminomonaspaucivorans* gen. sp. nov, a mesophilic, anaerobic, amino-acid-utilizing bacterium. *Int. J. Syst. Evol., Microbiol*. 49:975-982.

Beller H.R., Spormann A.M., Sharma P.K., Cole J.K., Reinhard M (1996). Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacteria. *Appl. Environ. Microbiol.,* 62:1188-1196.

Brand T.P.H., Roest K., Chen G.H., Brdjanovic D., Loosdrecht M.C.M (2015). Occurrence and activity of sulphate reducing bacteria in aerobic activated sludge systems. *J. Microbiol. Biotechnol.,* 31(3):507-516.

Bryant M.P. (1972) Commentary on the Hungate technique for culture of anaerobic bacteria. *Amer. J. Clin. Nutr.,* 25:1324-1328.

Colin Y., Urriza M., Gassie C., Carlier E., Monperrus M., Guyoneaud R (2017). Distribution of sulfate reducing communities from Estuarine to Marine Bay Waters. *Microb. Ecol*., 73(1):39-49.

Cui J., Chen X., Nie M., Fang S., Tang B., Quan Z., Li Bo., Fang C (2017). Effects of *Spartina alterniflora*, Invasion on the abundance, diversity, and community structure of sulfate reducing bacteria along a successional gradient of costal salt marshes in China. *Wetlands*., 37(2):221-232.

Enning D., Garrelfs J (2014). Corrosion of iron by sulfate-reducing bacteria: new views of an old problem. *Appl. Environ. Microbiol.,* 80-4:1226-1236.

Fauque G.D (1995). Ecology of sulfate-reducing bacteria. Biotechnology Handbooks. 8:217-241.

Foti M., Sorokin D.Y., Lomans B., Mussman M., Zacharova E.E., Pimenov N.V., Kuenen J.G., Muyzer G (2007). Diversity, activity, and abundance of sulfate-reducing bacteria in saline and hypersaline soda lakes. *Appl. Enviorn. Microbiol.,* 73(7):2093-2100.

Gallagher K.L., Kading T.J., Braissant O., Dupraz C., Visscher P.T (2012). Inside the alkalinity engine: the role of electron donors in the organomineralization potential of sulfate-reducing bacteria. *Geobiology.,* 10(6):518-530.

Hansen L.S., Blackburn T.H (1995). Amino acid degradation by sulfate-reducing bacteria: evaluation of four methods. *Limnol. Oceanogr.,* 40(3):502-510.

He Y., Xiao X., Wang F (2013). Metagenome reveals potential microbial degradation of hydrocarbon coupled with sulfate reduction in an oil-immersed chimney from Guaymas Basin. *Front. Microbiol.,* 4:148.

Howard B.H., Hungate R.E (1976). *Desulfovibrio* of the sheep rumen. *Appl. Environ. Microbiol.,* 32:596-602.

Hungate R.E (1969). A roll tube method for cultivation of strict anaerobes, In Norris J.R and Ribbons D.W (ed.), (Academic Press, New York). *Methods in Microbiology.* 3B:117-132.

Joblin K.N (1999). Ruminalacetogens and their potential to lower ruminant methane emissions. *Aust. J. Agric. Res.,* 50(8):1307-1314.

Kaur R., Anish D (2016). Assessment of water quality of Tung Dhab drain-an international water channel-using multivariate statistical techniques. *Asian J. Water Environ.,* 13(3):85-93.

Kharrat H., Karry F., Bartoli M., Hnia W., Mhiri N., Fardeau M., Bennour F., Kamoun L.N., Alazard D., Sayadi S (2017). *Desulfobulbusaggregans* sp. nov, a novel sulfate reducing *bacterium* isolated from marine sediments from Gulf of Gabes. *Curr. Microbiol*., 74(4):449-454.

Kuppardt A., Kleinsteuber S., Vogt C., Luders T., Harms H., Chatzinotas A (2014). Phylogenetic and functional diversity within toluene-degrading, sulphate-reducing consortia enriched from a contaminated aquifer. *Microb. Ecol.,* 68(2):222-234.

Leavitt W.D., Cummins R., Marian M.L., Sim M.S., Ono S., Bradley A.S., Johnston D.T (2014). Multiple sulfur isotope signatures of sulfite and thiosulfate reduction by the model dissimilatory sulfate-reducer, *Desulfovibrioalaskensis* str. G20. *Front. Microbiol.,* 5:591.

Magot M (2005). Indigenous microbial communities in oil fields, In Ollivier B and Magot M (ed.), Petroleum Microbiology. ASM Press, Washington, DC 21-33.

Miller T.L., Wolin M.J (1973). Formation of hydrogen and formate by *Ruminococcusalbus*. *J. Bacteriol.,* 116:836-846.

Muller A.L., Kjeldsen K.U., Rattei T., Pester M., Loy A (2014). Phylogenetic and Environmental diversity of DsrAB-type dissimilatory (bi) sulfite reductases. *ISME. J.,* 9(5):1152-1165.

Parshina, S.N., Sipma J., Nakashimada Y., Henstra A.M., Smidt H., Lysenko A.M., Lens P.N.L., Lettinga g., Stams A.J.M (2005). *Desulfotomaculum carboxydivorans* sp. nov, a novel sulfate-reducing bacterium capable of growth at 100% CO. *Int. J. Syst. Evol. Microbiol.,* 55:2159-2165.

Pimenov N.V., Zakharova E.E., Bryukhanov A.L., Korneeva V.A., Kuznetsov B.B., Tourova T.P., Pogodaeva T.V., Kalmychkov G.V., Zemskaya T.I (2014). Activity and structure of the sulfate-reducing bacterial community in the sediments of the southern part of Lake Baikal. *J. Microbiol.,* 83(1-2):47-55.

Pitcher D.G., Saunders N.A., Owen R.J (1989). Rapid extraction of bacterial genomic DNA with guanidiumthiocyanate. *Lett. Appl. Microbiol.,* 8:151-156.

Postgate J.R (1963). Versatile medium for the enumeration of sulfate-reducing bacteria. *Appl. Microbiol.,* 11(3):265-267.

Rabus R., Hansen R.T.A., Widdel F (2013). Dissimilatory sulfate and sulfur-reducing prokaryotes. The Prokaryotes (ed.), E. Rosenberg *et al* (Springer Verlag, New York). 309-404.

Rabus R., Hansen T.A., Widdel F (2003). The Prokaryotes (ed.), Dworkin M., Schleifer K.H., Stackebrandt E (Springer Verlag, New York). 659-768.

Tanimoto Y., Bak F (1994). Anaerobic degradation of methylmercaptan and dimethyl sulfide by newly isolated thermophilic sulfate-reducing bacteria. *Appl. Environ. Microbiol.,* 60:2450-2455.

Winfrey M.R., Ward D.M (1983). Substrate for sulfate reduction and methane production in intertidal sediments. *Appl. Environ. Microbiol.,* 45:193-199.

Xia F.F., Su Y., Wei X.M., He Y.H., Wu Z.C., Ghulam A., He R (2014). Diversity and activity of Sulphur-oxidizing Bacteria and Sulphate-reducing Bacteria in Landfill

cover soils. *Lett. Appl. Microbiol.,* 59(1):26-34.

Yang K., Zhu Y., Shan R., Shao Y., Tian C (2017). Heavy metals in Sludge during anaerobic Sanitary Landfill: Speciation Transformation and Phytotoxicity. *J. Environ. Manage*, 189:58-66.

Zobell C.E., Rittenberg SC (1948). Sulfate-Reducing Bacteria in Marine Sediments. *J. Mar.Res.,*7:602-617

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