Original Research Article

Versatile medium for the isolation of sulfur-reducing bacteria from natural ecotypes

ABSTRACT

Sulfate-reducing bacteria (SRB) are a diverse group of microorganisms that are commonly isolated from anoxygenic environments (lake depths, soil, or swamps) and are also found in the intestines of humans and animals. They are well known for outcompeting methanogens for common substrates thereby helping in the decrease of methane emissions and also playing a major role in sulfur cycling. Therefore, determining the cultivable methods for their isolation is important. Various media has been used for the cultivation and purification of SRB from natural wetlands. The maximum growth of SRB was observed in all the media tested, out of which the best suitable media to recover a number of colonies from the natural soil/sediment samples was discovered as a modified postage medium. The enumeration of the isolated SRB was done by the most probable number (MPN) technique. A total of twenty pure isolates of SRB were isolated from different ecotypes.

Keywords; Sulfate-reducing bacteria (SRB), Anoxic environment, MPN Technique, Natural wetlands.

1. INTRODUCTION

Sulfate-reducing bacteria (SRB) are common in anaerobic environments where sulfate-containing substances are prevalent [1]. Therefore, SRBs are common in a variety of environments, including soils, marshes, lakes [2], and biogas plants [3]. They are also found in both human and animal intestines [4, 5]. The main substrates for methanogens and SRB are acetate and hydrogen [6]. SRB can use hydrogen and acetate at lower concentrations than methanogens. So, they will likely outcompete them for substrate uptake. This will direct the electron flow toward CO_2 production rather than methane production [7, 8].

These microbes utilize sulfate ions, which undergo a process known as "dissimilatory sulfate reduction" or "sulfate respiration" in which it is converted to hydrogen sulfide (H_2S). Sulfate serves as a terminal electron acceptor in this process [9]. Exogenous electron donors are required for dissimilatory sulfate reduction to be carried out [10]. The primary electron provider for all SRB is molecular hydrogen, other common electron donors include ethanol, lactate, acetate, pyruvate, amino acids, fatty acids, and dicarboxylic acids [11, 12]. Organic molecules present in the soil can either undergo complete oxidation to carbon dioxide or incomplete oxidation to acetate, depending on the microbial floral species (SRB) present in the soil. Ammonium salts are another source of nitrogen that the SRB can utilize. Molecular nitrogen can be assimilated by SRB species.

Methanogenesis is known to be constrained by sulfate reduction through a number of mechanisms. Sulfate supplementation or high sulfate concentrations will impede methanogenesis in complicated environments like natural sediments where sulfate-reducing bacteria and methanogens are present, directing the electron flow toward sulfate reduction[13, 14]. Therefore, it is required to isolate new SRB strains, purify them from other bacteria, and thoroughly examine the cultural, physiological, biochemical,

and genetic characteristics of SRB. There are various mediums found for the cultivation of SRB but inadequacies in the methods used to estimate the quantities of sulfate-reducing bacteria in natural environments have been noted [15]. The cysteine media suggested by [16] for culturing *Desulfovibrio desulfuricans* are inconvenient when used with natural samples because cysteine-decomposing microbes can cause blackening of colonies or media and thus give false positive results [17]. So, the best medium is needed to cultivate SRB from natural ecotypes.

2. MATERIALS AND METHODS

2.1. SAMPLING

Sediment samples were collected aerobically by flushing N_2 gas with the bladder from various Rivers, Ponds rice fields, Mangroves, and Aquaculture sediments in different states of Andhra Pradesh, West Bengal, and Tamil Nadu (Table 2). The sediment samples collected were immediately transported to the laboratory and proceeded with isolation and the rest were stored at 4 ºC.

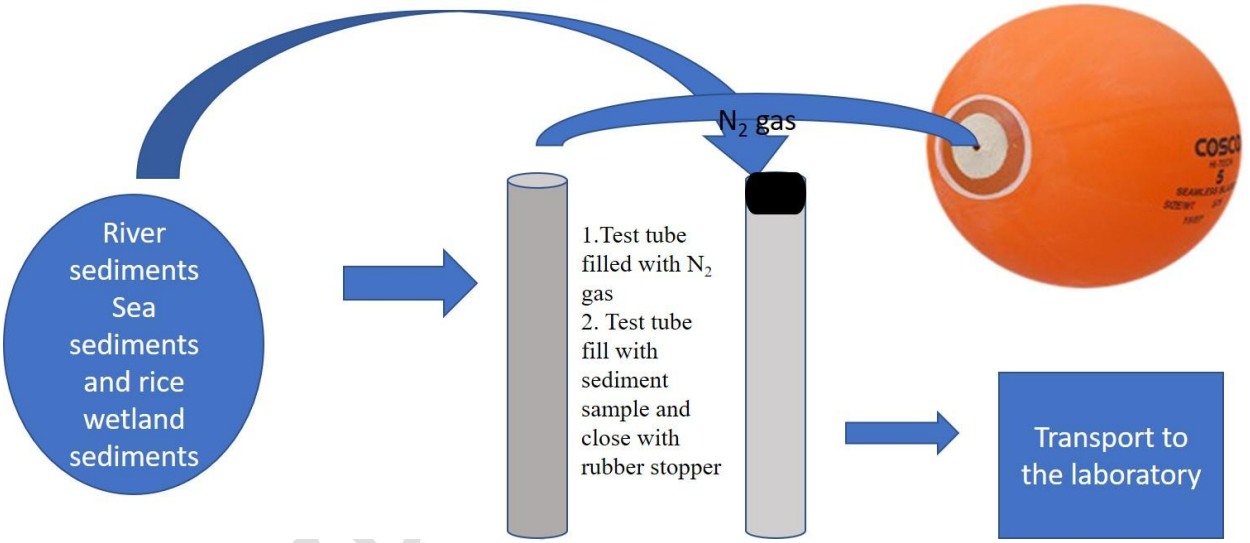

Fig. 1. Schematic diagram illustrating collection of soil/sediment samples from anoxic ecosystem

2.2. MEDIA PREPARATION AND ENUMERATION OF SRB AND PURIFICATION

Under anoxic conditions, the required amount of dilution buffer was prepared. To maintain anoxic conditions, dilution buffer was dispensed at a rate of 9 ml in serum bottles flushed with N_2 gas and crimped airtight. The crimped vials were autoclaved at 121°C at 15 psi pressure for 15 to 20 minutes before being used for dilution. Under anoxic conditions, media was prepared by flushing N_2 gas through a gassing manifold. SRB was isolated using four different types of media (shown below along with their composition) and further purification was done in modified Postage's media, The prepared media was then autoclaved for 15 minutes at 121°C. Following autoclaving, a sterile syringe fitted with a 2mm syringe filter was used to add vitamin solution to the media under anoxic conditions prior to inoculation in an oxygen-free N_2 atmosphere. Under N_2, the sample was serially diluted by adding the collected soil sample to sterilized serum vials containing 9 ml of dilution buffer. The medium was then mixed with 1 ml of sterile vitamin solution and trace element solution in the presence of O_2-free N_2 gas, which was continuously flushed using a gassing manifold. The sterilized roll tubes were then filled with 1 ml of the diluted sample from the dilution buffer and 9 ml of broth in an O_2-free N_2 gas atmosphere maintained by a

gassing manifold. Finally, the test tubes are incubated in an anaerobic gas jar at 37°C till the development of black color. The four different types of media selected for the cultivation of SRB were as follows

Media 1 (g/l) : K_2HPO_4 -0.5; NH_4Cl- 1; $CaSO_4$-1; $MgSO_4.7H_2O$-2; sodium lactate (70% solution)- 5; $(NH_4)_2$ Fe $(SO_4)_2.6H_2O$-0.5 ; tap water, 1 1; pH 7.0-7.5[18].

Media 2 (g/l): K_2HO_4-0.5; NH_4Cl- 1; Na_2SO_4- 1; $CaC1_2$. $2H_2O$- 0.1; $MgSO_4.7H_2O$- 2; sodium lactate (70% solution)- 5; $(NH_4)_2$ Fe $(SO_4)_2.6H_2O$-0.5; distilled water-1 1[19].

Media 3 (g/l): K_2HPO_4 -0.5 g; NH_4Cl- 1; Na_2SO_4- 1; $CaC1_2$. $2H_2O$- 0.1. $MgSO_4.7H_2O$- 2 g; sodium lactate (70% solution)- 5; Difco yeast extract-1 g; $(NH_4)_2$ Fe $(SO_4)_2.6H_2O$-0.5; distilled water-1 1[20].

Media 4 Modified Postgate medium [21, 22] (g/l): Na_2SO_4 -0.5 ; KH_2PO_4 -0.3; K_2HPO_4 - 0.5; $(NH_4)_2SO_4$ - 0.2; NH_4Cl -1.0; $CaCl_2$.6H2O -0.06; $MgSO_4$7.H2O -0.1; sodium lactate, $C_3H_5O_3Na$ -2.0; yeast extract 1.0; $FeSO_4$ .7H2O -0.004; sodium citrate-0.3; and distilled water (1 l).**Separated solutions**: Mohr's salt solution ($(NH_4)_2Fe(SO_4)_2$.$6H_2O$) (10%) and Na_2S . $9H_2O$ solution (1%) and 10 M solution of NaOH must be sterilized separately.

3. Results and discussion

The preparation of an anaerobic medium for the cultivation and isolation of pure SRB is a gargantuan process [23]. There are a number of media for their cultivation. But the common problem encountered with these media is precipitation issues during the preparation of media, and another major problem is the blackening of the entire media due to bacterial production of hydrogen sulfide (H_2S) which interacts with the iron present in the medium and it precipitates. The other reason is contamination with H_2S-producing satellite microorganisms. The stichometry of precipitation with iron as follows as shown in the equation-1

$$Fe^{+2} + H_2S \rightarrow FeS + 2H^+ \rightarrow 1$$

Previously various media has been employed for the cultivation of SRB based on the species. For the isolation of SRB from the natural ecosystem, four different types of media have been selected based on the previous findings. Sediment samples from various sources were sampled onto four different types of media. The best was selected based on the greater number of MPN log cfu/g^{-1} of soil obtained. The four different types of media employed were as follows given by [18–22]. Most-probable-number (MPN) method was used for the selective enumeration of sulfate-reducing bacteria (SRB) followed by the method given by [24]. SRB densities were determined in sediment samples using a normal MPN (N-MPN). Results show that all the tested media are capable of recovering colonies of SRB from their natural habitat among them the best media was discovered as a modified Postage medium for the isolation of SRB from natural ecosystems obtaining the highest number of MPN log cfu/g^{-1} of soil. The values of the MPN table obtained by the cultivation of SRB were depicted in Table 1. In the bars, medium sulfate source is taken from the tap water. Where the bacteria utilize more sulfur sources from natural supplements rather than artificial chemical supplements. These can correspond to more MPN log cfu/g^{-1} of soil than starkey and waring medium in bars medium. The other major change in these media is the addition of yeast extract in waring and modified postage medium which is a good source of nitrogen and is one of the main reasons for the luxuriant growth of SRB in the modified postage medium. This is also one of the main reasons for a greater number of MPN log cfu/g^{-1} of soil in modified postage medium. This also determines their heterotrophic behavior. The various types of media evolved for the cultivation of sulfur-reducing bacteria based on the habitat. The modified postage medium recipe enriched with all the conditions required for the growth similar to that of natural habitat is one of the main reasons for the recovery of more colonies from the modified postage medium which shows higher colonies from all the

sediment samples shown in Table 1. Despite the fact that the same carbon source was used in all three mediums, each strain of SRB has a preferred medium for growth. These findings indicated that each chemical composition in the medium had a different effect on bacterial growth. Furthermore, the presence of iron in the medium is an important component that aids the microbial activity of SRB [25]. The locations from which samples are collected are shown in Table 2

Isolate code	Medium 1 10^3 Dilution	Medium1 10^4 Dilution	Medium2 10^3 Dilution	Medium2 10^4 Dilution	Medium3 10^3 Dilution	Medium3 10^4 Dilution	Medium4 10^3 Dilution	Medium4 10^4 Dilution
RKS	4.98×10^3	5.85×10^4	4.92×10^3	5.81×10^4	4.84×10^3	5.66×10^4	5.08×10^3	5.98×10^4
MS1	5.18×10^3	6.08×10^4	5.18×10^3	6.08×10^4	4.98×10^3	5.85×10^4	5.38×10^3	6.34×10^4
MS2	5.18×10^3	6.08×10^4	5.18×10^3	6.08×10^4	4.98×10^3	5.85×10^4	5.38×10^3	6.34×10^4
PPS	5.23×10^3	6.11×10^4	5.32×10^3	6.15×10^4	4.85×10^3	5.69×10^4	5.41×10^3	6.34×10^4
SPS	5.32×10^3	6.26×10^4	5.26×10^3	6.18×10^4	4.98×10^3	5.85×10^4	5.45×10^3	6.34×10^4
M1S	4.91×10^3	5.86×10^4	4.81×10^3	5.75×10^4	4.56×10^3	5.51×10^4	5.26×10^3	6.18×10^4
CDS	4.68×10^3	5.75×10^4	4.73×10^3	5.67×10^4	4.6×10^3	5.53×10^4	5.34×10^3	6.08×10^4
SGS	5.34×10^3	6.38×10^4	5.23×10^3	6.11×10^4	5.18×10^3	5.98×10^4	5.41×10^3	6.32×10^4
WCS1	5.23×10^3	6.11×10^4	5.04×10^3	5.91×10^4	4.91×10^3	5.86×10^4	5.45×10^3	6.34×10^4
WCS2	5.23×10^3	6.11×10^4	5.04×10^3	5.91×10^4	4.91×10^3	5.86×10^4	5.54×10^3	6.45×10^4
RWS	5.32×10^3	6.26×10^4	5.26×10^3	6.15×10^4	5.23×10^3	6.11×10^4	5.45×10^3	6.41×10^4
PPS	5.26×10^3	6.18×10^4	5.15×10^3	5.9×10^4	5.04×10^3	5.85×10^4	5.41×10^3	6.32×10^4
SMS1	5.23×10^3	6.18×10^4	5.11×10^3	6.04×10^4	4.98×10^3	5.85×10^4	5.45×10^3	6.34×10^4
SMS2	5.23×10^3	6.18×10^4	5.11×10^3	6.04×10^4	4.98×10^3	5.85×10^4	5.45×10^3	6.34×10^4
KPS	4.91×10^3	5.86×10^4	4.81×10^3	5.61×10^4	4.59×10^3	5.52×10^4	5.45×10^3	6.34×10^4
VPS	4.81×10^3	5.75×10^4	4.65×10^3	5.61×10^4	4.53×10^3	5.59×10^4	5.34×10^3	6.23×10^4
GCS	4.86×10^3	5.81×10^4	4.57×10^3	5.51×10^4	4.51×10^3	5.45×10^4	5.15×10^3	6.04×10^4
MRS	4.91×10^3	5.86×10^4	4.81×10^3	5.68×10^4	4.73×10^3	5.61×10^4	5.45×10^3	6.41×10^4
TKS	4.84×10^3	5.79×10^4	4.73×10^3	5.67×10^4	4.65×10^3	5.57×10^4	4.91×10^3	5.81×10^4
PRS	4.98×10^3	5.88×10^4	4.75×10^3	5.68×10^4	4.65×10^3	5.61×10^4	5.26×10^3	6.15×10^4
SE(d)	0.097	0.126	0.093	0.120	0.117	0.126	0.114	0.105
CD(0.05)	0.196	0.256	0.189	0.243	0.238	0.225	0.231	0.213

Table. 1. MPN log cfu/g^{-1} of SRB obtained after growth on four different types of media where the highest values of colony forming units per gram of soil (cfu/g^{-1}) were recorded in the media 4 (modified postage medium).

3.1. ISOLATION OF PURE CULTURES

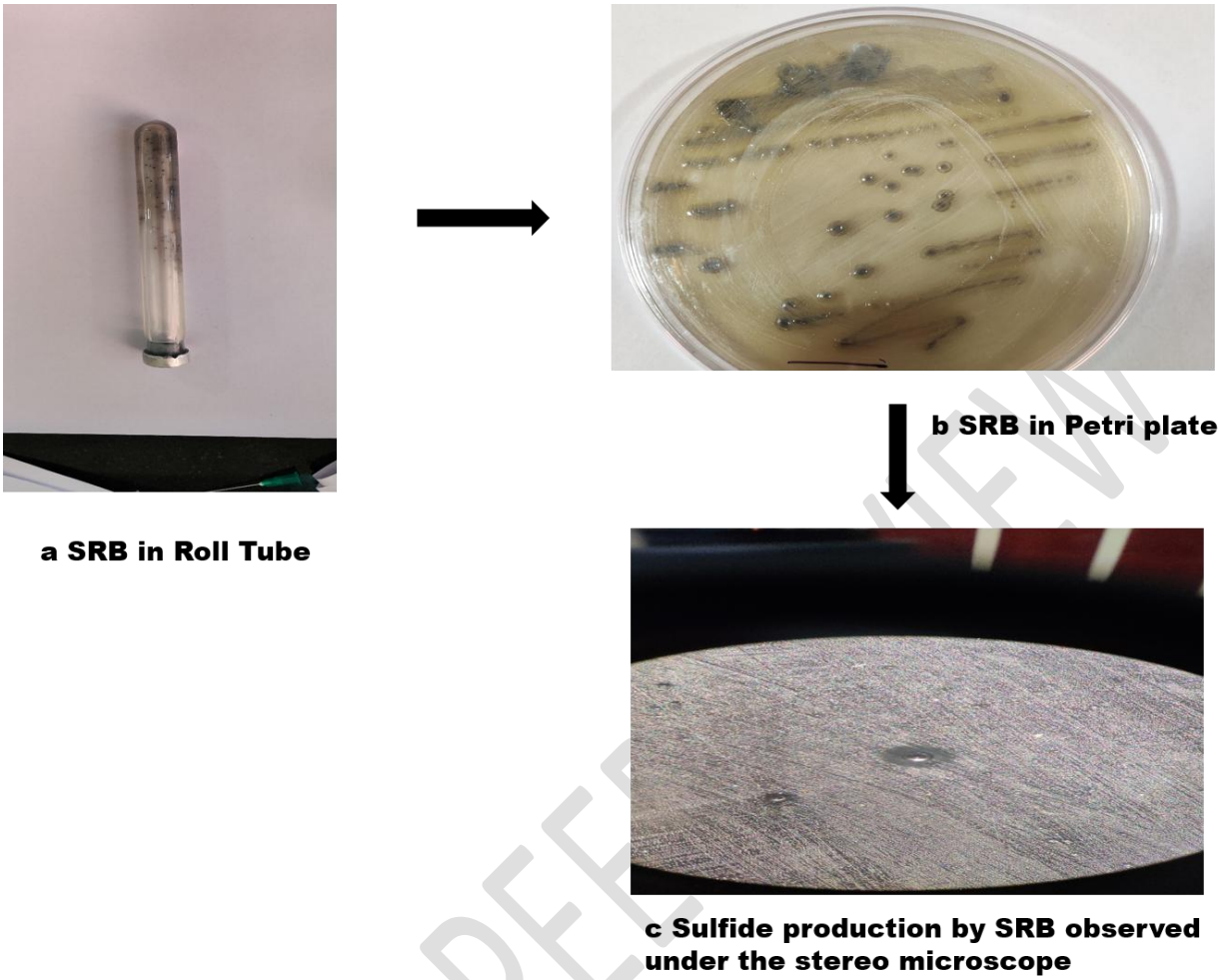

Fig. 2. Pure culture of sulfur-reducing bacteria 2a. pure single colonies of SRB in roll 2b. pure cultures of SRB in modified postage medium plate depicting black colonies 2c.SRB shows the production of sulfide observed beneath the stereomicroscope.

SRB interact with other microorganisms and can form biofilms with which they may have a symbiotic relationship. Such microorganisms cooperating with SRB are often called satellite microorganisms [26]. Before beginning, preparation of modified Postgate agar medium with the same composition as liquid, but this time add additional compounds to the medium: Na_2SO_3 (3%) and microbiological agar (12 g/l). Autoclave it like Postgate liquid medium to sterilize it. Most intestinal species of the Enterobacteriaceae family, including Bacteroides, Pseudomonas, Clostridium, and Escherichia, which can be satellites of SRB, are inhibited by sodium sulfite at high concentrations in the medium [27]. Because they are sensitive to sulfite reductase activity, SRB species are resistant to sulfite ions and can be used as an alternative electron acceptor in the process of dissimilatory sulfate reduction [28, 29]. After sterilization in an autoclave, the modified Postgate agar medium containing sodium sulfite (Na_2SO_3) should be cooled to 40°C and 10 ml/l of sterile Mohr's salt solution, 0.05 ml/l of sterile sodium sulfide and ascorbic acid (0.1 g/l) added to the medium These components must be thoroughly mixed in the flask before adding a sterile 10 M solution of NaOH to provide a pH appropriate for the samples. To keep the medium from solidifying, use a water bath to maintain a constant temperature (40°C). In total, spill 20 ml of warm modified Postgate agar medium in Petri dishes and add 1ml of each diluted suspension of a positive sample to the medium, thoroughly mixing the suspension with the warm medium. The temperature should

be according to the sample from where it was isolated. Petri plates are placed in an anaerobic box with oxygen uptake sachets to facilitate anaerobiosis. Mohr's salt agar medium allows the detection of black colonies of SRB because FeS was formed as a result of hydrogen sulfide bacterial production, resulting in black colonies. Cultivate at the appropriate temperature in the thermostat. Depending on the sample and dilution, the black colonies will appear in 1-7 days in the deep agar medium. The entire plate will turn black color (fig 2) pick a single colony and purify the same as following the above procedure a total of 20 pure SRB was isolated from different ecotypes as shown in Table 2. Cysteine HCL cannot be used as a reducing agent because the active compound of cysteine-HCl is cysteine and at neutral pH values, the thiols of two cysteines react with oxygen resulting in the disulfide cystine and water which interacts with iron gives false results denoting the presence of SRB. Sodium thioglycolate can be used as a reducing agent.

Isolate code	Location co-ordinates	Colony morphology	Gram reaction	H_2S
RKS	Reddikunta lake (Andhra Pradesh) (14.183935 º N/ 78.695247 º E)	Tiny, round transparent colonies formed a black zone around them, turning the whole slant black within 2 days of growth	Gram-negative	Positive
MS1	Pichavaram (11.417586 º N/79.772133 º E)	Medium, Round, transparent, smooth, deep black colonies	Gram-positive	Positive
MS2	Pichavaram (11.417586 º N/79.772133 º E)	Tiny, round, pin-pointed, black,	Gram-negative	Positive
PPS	Parangipettai (11.292611 º N/79.455708 º E)	Medium, round, smooth, black colonies.	Gram-negative	Positive
SPS	Samiyarpettai (11.551264 º N/79.759134 º E)	Medium, Round, transparent, smooth, deep black colonies	Gram-negative	Positive
M1S	Aquaculture sediments	Small, round, transparent black colonies.	Gram-negative	Positive
CDS	Chidambaram (11.406645 º N/79.691559 º E)	Medium, round, smooth, black colonies.	Gram-negative	Positive
SGS	Silambimangalam (11.538645 º N/79.762559 º E)	Medium, round, smooth, black colonies.	Gram-negative	Positive
WCS1	Winogradsky column sediments	Round, medium, transparent,	Gram-positive	Positive

		smooth, deep black colonies		
WCS2	Winogradsky column sediments	Small black round colonies	Gram-negative	Positive
RWS	Wetland (11.002288 º N/ 76.926175 º E)	Black tiny small colonies	Gram-negative	Positive
PPS	Poosaripalayam (11.004039 º N/ 76.932391 º E)	Small black tiny colonies	Gram-negative	Positive
SMS1	Sundarban Mangroves (22.308039 º N/ 88.662991 º E)	Small round black colonies	Gram-negative	Positive
SMS2	Sundarban Mangroves (22.308039 º N/ 88.662991 º E)	Tiny black colonies	Gram-negative	Positive
KPS	krishnampathy lake (11.004363 º N/ 76.925233 º E)	Medium size black colonies	Gram-negative	Positive
VPS	Velingarayan Pettai (11.321652 º N/ 79.454573 º E)	Black colored colonies	Gram-negative	Positive
GCS	Golden Cheruvu (14.183254 º N/ 78.699452 º E)	Black-shaped colonies entire roll tube	Gram-negative	Positive
MRS	Mandavya river (14.058619 º N/ 78.751989 º E)	Small black colonies	Gram-negative	Positive
TKS	Thimaya kunta (14.173931 º N/ 78.685244 º E)	Small black colonies	Gram-negative	Positive
PRS	Perur pond (10.964691 º N/ 76.930098 º E)	Black shaped colonies	Gram negative	Positive

Table. 2. Brief characteristics of sulphur reducing bacteria isolated from different natural ecotypes

4. CONCLUSION

In the largest context, obligate anaerobes are microbes that are incapable of growing in the presence of molecular oxygen. Significant levels of oxygen in the atmosphere only slightly inhibit aerotolerant anaerobes. There are numerous strains and possibly species of sulfate-reducing bacteria. They can be isolated using a variety of media over a wide temperature range. Although crude cultures are easily

obtained, but isolating absolutely pure cultures is typically difficult. Research findings made it easier for the cultivation and purification of SRB from natural ecosystems.

ETHICAL APPROVAL

Not applicable.

CONSENT FOR PUBLICATION

All authors have reviewed the manuscript and agree to its publication.

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