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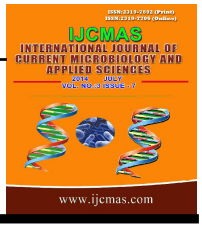

Original Research Article

Isolation and Identification of Sulfate Reducing Bacterial Strains Indigenous to Sulphur Rich Barite Mines

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A B S T R A C T

The present study was aimed at isolating Sulfate Reducing Bacteria(SRB) by enriched method using Iron-Lyngby medium from barium sulphate mines situated in Mangampeta, Kadapa district of Andhra Pradesh. Isolation of bacterial strains was done by dilution plate technique continued by three tier identification process such as morphological, microscopic studies and biochemical characterization. Identification was done using biochemical tests such as, ONPG, Lysine utilization, Ornithine utilization, Urease production, Phenylalanine Deamination, Nitrate reduction, H_2S production, Citrate Utilization, Voges Proskeur's reaction, Methyl red, Indole production, Malonate utilization, and Esculin hydrolysis. Carbohydrate utilization potentials were also tested using Arabinose, Xylose, Adonitol, Rhamnose, Cellobiose, Melibiose, Saccharose, Raffinose,Trehalose, Glucose and Lactose. In total five species of bacterial strains *viz*., four species were belongs to the *Enterobacter species* and one *species* of *Bacillus* were identified from the barite mine sample. Physiological responses were also studied for these strains in different pH, Nacl concentration and temperatures. These strains have potential use in bioremediation of sulphur contaminated environments.

Keywords

Sulfate Reducing Bacteria (SRB), Iron-Lyngby medium, Barite mine, Barium sulphate, *Enterobacter species* and *Bacillus species*

Introduction

Microbial diversity is rapidly gaining interest among the scientific community with emphasis to understand their eco physiological role and function in various ecosystem. The role of microorganisms in an ecosystem is influenced by ecosystem structure, composition, nature and location. The Sulfate reducing bacteria (SRB) are a heterogeneous group of microbes which use sulfate as terminal electron acceptor (Hansen, 1994). They use simple inorganic and organic compounds like hydrogen, ethanol, methanol, acetate, lactate, propionate, and pyruvate as electron donor (Liamleam and Annacchatre, 2007). The sulphate reducing bacteria (SRB) are unique physiological group of prokaryotes because they have capability of using sulphate as the final electron acceptor in respiration. A hall mark characteristic that distinguish SRB is the manner in which sulphate is metabolized. The sulphate reducing bacteria

are one of the wide technological interest not only for their ability to reduce metal sulphide but also for formation of insoluble metal sulfide thus removing toxic metals from waste water (Hoa *et al*., 2007; Biswas *et al*., 2008).

Barite ($BaSO_4$) is a sulphur rich deposit generally arising from mixing of soluble Barium containing fluids with sulphate rich fluids. Abiotic oxidation of sulphide to sulphate leads to barite deposition (Plummer, 1971). Alternatively barium leads brines may mix barite formation (Kasiser, 1987; Williams Jones *et al*, 1992).Basically barium is an alkaline earth element which occurs as a trace metal in igneous and sedimentary rocks. In nature it occurs principally in combined states as Barite ($BaSo_4$) and Witherite ($BaCo_{3)}$. Barium precipitates as the mineral barite(barite). Even though recent investigations have shown that Barium (Ba) may serve as an indicator of palio oceanographic and modern conditions, a better understanding of its biogeochemistry is required before confident interpretation of its distribution and reaction ways are possible. Barium has a low solubility from Barite but it has been observed that laboratory cultures of sulphate bacteria have been indicated as good candidates for barium solubilisation from barite. The present study intends to isolate SRB which will help to mitigate the toxic effects of Ba in Eukaryotic and Prokaryotic cells.

Materials and Methods

Study area

The bedded barite deposits are located at Mangampeta (Lat. $14^0.01'$ N; long 70^0 $19'$E) in Kadapa district, Andhra Pradesh (Fig-1). It is included in the survey of India Topo sheet No.57 N/8. This is the single largest deposit of its kind in the world. The mining activity is generally conducted as opencast and underground mining. Sulfur Content is high in this area.

Collection of sample

Barite mine samples have been collected from different operating opencast mines. The samples collected were from old ore deposits using randomised block design. The collected samples were placed in sterile poly bags and brought to the laboratory and stored at 4^0C until further processing.

Isolation and pure culture preparation

Isolation of native microorganisms present in the mine samples were done by selective isolation and enrichment method using Iron Lyngby *Medium* (Lorentzen et al., 2003). This medium is generally used for the isolation of sulphur oxidizing bacteria and the ingradients of the medium are Peptone - 20g/l; Yeast Extract - 3g/l; Ferric Citrate - 0.3g/l; Sodium Thiosulphate - 0.3g/l; NaCl-5g/l at pH 7.5. Isolation of native microorganisms present in the mine samples were done by dilution plate technique. From the cultured plates the morphologically different strains were isolated and grown on the same medium for pure culture preparation. The plates were incubated at 37^0C for 24hr. Pure cultures were preserved in Glycerol medium and stored at -20^0C.

Morphological and Microscopic studies

The colony characteristics were studied basing on their shape, size, elevation, margin, surface, colour and structure of the colony. Microscopic studies of the isolated strains were done by Gram staining for identification of structural characteristic and Gram '+'ve or Gram '-'ve nature.

Biochemical studies

Various biochemical tests were carried out

to characterize the isolated bacterial strains. The tests include ONPG, Lysine utilization, Ornithine utilization, Urease production, phenylalanine deamination, nitrate reduction, H_2S production. Citrate utilization Voges Proskauer's reaction, Methyl red, Indole prodduction, Malonate utilization, Esculine hydrolysis, Oxidase production, Arabinose, Xylose, Adonitol, Rhamnose, Cellobiose, Melibiose, Saccharose, Raffinose, Trehalose, Glucose and Lactose utilizations.

Results and Discussion

From the distinct isolated colonies in the petri plates five different strains were selected on the basis of distinct morphological characteristics such as colony shape, size, margin, colour and elevation as given Table-1. The selected strains were sub cultured repeatedly several times to obtain the pure culture and named as CSRB-1, CSRB-2, CSRB-3, CSRB-4, CSRB-5. These pure strains were subjected to Microscopic, biochemical and growth characteristics. The Gram staining was carried out for five bacterial strains to differentiate between two principal groups of bacteria *i.e.,* Gram positive and Gram negative nature and their shape such as *Bacilli* or *Cocci* etc., were studied. Further, various biochemical tests related to the characterization of bacteria were carried out to identify them (Table-2). The carbohydrate utilization profiles were also studied. The isolate CSRB-1 was found to be circular in shape, cream in colour and the margin was lobate having crenate elevation. The colony size after 24 hours incubation was measured as 0.1 - 1.2mm. This isolate was found to be Gram negative, rod shaped bacteria. From the biochemical tests it was found that the bacteria is facultative anaerobe. The isolate also showed positive for Urease production, Ornithine Utilization, Methyl red test and nitrate reduction and negative for ONPG, Lysine utilization, Phenylalnine deamination, H_2S production, Citrate utilization, Voges Proskauer's reaction, Indole production, Malonate utilization, and Esculin Hydrolysis. The isolate was able to utilize Cellobiose, Melibiose, Saccharose, Raffinose, Trehalose and Glucose and unable to utilize Arabinose, Xylose, Adonitol, Rhamnose and Lactose.

Due to these results the isolate was identified as *Enterobacter sp.*. The isolate CSRB-2 was found to be circular in shape, yellow in colour and margin was entire having flat elevation. The colony size after complete incubation was measured as 0.1 – 1.5 mm. The isolate was found to be Gram negative rod shaped bacteria. Biochemical tests showed positive for Nitrate reduction, Methyl red and Esculin hydrolysis and negative for ONPG, Lysine utilization, Ornithine utilization, Phenylalnine deamination, H_2S production, Citrate utilization, Voges Proskauer's reaction, Indole production, and Malonate utilization. The carbohydrates Saccharose, Trehalose, and Glucose are utilized by the isolate. But, Arabinose, Xylose, Adonitol, Rhamnose, Cellobiose, Melibiose, Raffinose and Lactose were not utilized by the isolates. Due to the presence/absence of the above results the isolate was identified as *Bacillus sp.* The isolate CSRB-3 was found to be circular in shape, white in colour and the margin was lobate having umbonate elevation. The isolate was positive for Nitrate reduction, H_2S production, Methyl red, Esculin hydrolysis and negative for ONPG, Lysine utilization, Urease, phenylalanine deamination, Citrate utilization, Voges Proskauer' reaction, Indole, Malonate utilization and unable to utilize Arabinose, Xylose, Adonitol, Rhamnose, Cellobise, Melibiose, Raffinose, and Lactose. So isolate was identified as *Enterobacter sp.*

Table.1 Biochemical Characteristics of Sulfate reducing Bacteria

S.No	Name of the Test	CSRB-1	CSRB-2	CSRB-3	CSRB-4	CSRB-5
1	Gram Staining					
2	ONPG	-	-	-	-	-
3	Lysine Utilization	-	-	-	-	-
4	Orthinine Utilization	-	-	-	-	-
5	Urease	+	-	-	+/-	+/-
6	Phenylalanine Deamination	-	-	-	-	-
7	Nitrate Reduction	+	+	+	+	+
8	H2s Utilization	-	-	+	+	-
9	Citrate Uilization	-	-	-	-	-
10	Voges Proskauer's	-	-	-	+/-	-
11	Methyl Red	+	+	+	-	+
12	Indole	-	-	-	-	-
13	Malonite Utilization	-	-	-	-	-
14	Esculin Hydrolysis	-	+	+	+	+

Table.2 Carbohydrate Utilization Potentials of sulfate Reducing Bacteria

S.No	Name of the Carbohydrate	CSRB-1	CSRB-2	CSRB-3	CSRB-4	CSRB-5
1	Arabinose	-	-	-	+	-
2	Xylose	-	-	-	-	-
3	Adonitol	-	-	-	-	-
4	Rhamnose	-	-	-	-	-
5	Cellobiose	+/-	+/-	-	+	+
6	Melibiose	+/-	-	-	-	-
7	Saccharose	+/-	+	+	+	+
8	Raffinose	+/-	-	-	+	-
9	Trehalose	+	+	+	+	+
10	Glucose	+	+	+	+	+
11	Lactose	-	-	-	+	-
12	Oxidase	-	-	-	-	-

The isolate CSRB-4 was found to be circular in shape, cream in colour and the margin was lobate having flat elevation. The colony size after complete incubation was measured as 0.1 – 1.5µm. This isolate was found to be Gram negative. The result of biochemical tests positive for Nitrate reduction, H_2S production, Esculin hydrolysis and able to utilize Arabinose, Cellobiose, Saccharose, Raffinose, Trehalose, Glucose, and Lactose, so it comes under the genera *Enterobacter sp.*

The isolate CSRB-5 was found to be circular in shape, yellow in colour and the margin was entire having flat elevation. The colony size was after complete incubation was measured as Gram

negative rods. Due to yellow colour pigment production it comes under E*ntrerobacteriaceae* family. The biochemical tests include H_2S production, methyl red, Esculin hydrolysis and able to utilize Cellobiose, Saccharose, Trehalose, Glucose. Therefore the isolate was identified as *Enterobacter sp.*

Morphological, cultural, physiological and biochemical characteristics of bacterial isolates from different environments have been studied by various workers (Gahan *et al.*,2005, Dhal and Thatoi, 2007, Elizabeth *et al*.,2008). Venugopal *et al*., 2000 suggested that application of indigenously available microbes has immense potential as bioremediating agents. The organisms isolated from barite mines also would have immense potential applications in bioremediation of sulphur or desulpharization process. Lakshman raj *et al*., (2012) isolated the sulfur specific microorganisms from Indian refinery plant sites and used in desulfurization process. In his studies he identified *Enterobacter sp*., and *Pseudomonas sp.*, Similarly, *Bacillus species* were also isolated from oil contaminated soil and used in Biodiesulfurization (Kalyani *et al*.,2012) .Philip *et al*., (1998) found that *Bacillus* species were also involved in heavy metal reductions. Bacterial reduction of heavy metal by *Enterobacter* has also been reported (Ehrlich,1996; Shen,1950). The sulphur oxidizing bacteria are major potential applications in biodesulfurization, biodenitrogenation, biodemetallization , and biotransformation of heavy crude oils into lighter crude oils sulphur contaminated and heavy metal contaminated soils.

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