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Biosulphidogenesis: Isolation and characterization of Thermodesulfobacterium commune sp. nov. Isolated from hot water springs of Thane, Maharashtra.

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Abstract: *Biosulphidogenesis was examined in thermal waters, sediments and decomposing bacterial mats associated with Vajreshwari & Ganeshpuri hot springs, Thane. In vitro studies demonstrated biological sulphide production from sulphate at temperatures higher than 50°C but less than 85°C, correlated with the presence of a unique sulphate-reducing bacterium (TSRP- Thermophilic Sulphate Reducing Prokaryote). It showed complete reduction of sulpahte in 20 hrs with negligible production of sulphide. This new species proliferated at temperatures above 50°C but below 85°C, and had an optimum growth temperature of 60°C in anaerobic condition (The anaerobic condition was maintained by overlaying the media with Paraffin oil). The organism was a small gram-negative, straight rod, utilized lactate as electron donors and sulphate as electron acceptors for growth and forming sulphide, indicating presence of dissimilatory sulphate reduction mechanism. Other unusual biochemical features of this extreme thermophile includes tolerance to 1.5 % NaCl, lacked catalase activity and non motile. Strain TSRP is described as the type strain of the species Thermodesulfobacterium commune identified by 16s r RNA sequence analysis and phylogenetic relationship was found in between different OTUS which were determined by CLUSTALW.*

The assessment of the effluents collected from 3 industries viz., Textile, Battery, Paper & pulp; within the city zone of Thane showed high concentration of sulphate. These effluents were subjected to sulphidogenesis by T. commune & there was a complete reduction of sulpahte in 25,24,22 hrs. respectively with negligible production of sulphide.

The study showed that T. commune can be used for treating effluent containing sulphate, thus important for bioremediation process with high efficiency of sulfate reduction rate and negligible production of sulphide.

Keywords: *Biosulphidogenesis, TSRP, Dissimilatory Sulphate reduction pathway, 16s rRNA*

I. Introduction

Life on the earth is dependent on balanced recycling of various elements like nitrogen, carbon, sulfur etc. Out of which sulphur is present in its most reduced form in biological material. During decomposition of this material, sulfide is oxidized to sulphate under aerobic conditions while during anaerobic condition sulphide is liberated as hydrogen sulfide (Christian Jeanthon, et.al.,2002). Another important source of hydrogen sulphide is dissimilatory sulphate reduction by anaerobic microorganism which uses sulphate as electron acceptor for oxidation of organic compound. Sulphate reducers play an important role in balancing the sulphur cycle.

Human activities have a major effect on the global sulfur cycle. The burning of coal, natural gas, and other fossil fuels has greatly increased the amount of S in the atmosphere and ocean and depleted the sedimentary rock sink. Sulphates or sulphuric acid products are also used in the manufacture of numerous chemicals, dyes, glass, paper, soaps, textiles, fungicides, insecticides, astringents and emetics. They are also used in the mining, pulping, metal and plating industries, in sewage treatment and in leather processing. These effluent containing sulphate are discharged into the aquatic body. The aesthetic objective for sulphate in drinking water is ≤500 mg/L, based on taste considerations. Because of the possibility of adverse physiological effects at higher concentrations, it is also recommended that health authorities be notified of sources of drinking water that contain sulphate concentrations in excess of 500 mg/L.

Many bacteria reduce sulpahte in large amount to produce energy and to expel sulfide as waste. These bacteria are known to be sulphate reducing bacteria (Anna H. Kaksonen,et.al.2006). Thermophilic bacteria from the hot water spring are adapted to the environment containing high amount of sulphate. Thus can serve as an important tool for biosulphidogenesis. The aim of the study was to isolate, characterize and identify thermophilic sulfate reducing prokaryote (TSRP) from Vajreshwari and Ganeshpuri hot springs, Thane, Maharashtra.

II. Materials and Methods

Sampling

Water samples were collected from seven different hot springs (from Vajreshwari and Ganeshpuri, Thane) Mumbai. Various parameters were noted down like temperature, pH, etc. the samples were taken into clean polyethylene container with lid. Water sample used for inorganic analyses was immediately fixed with zinc acetate solution as described in APHA (Patil S. Z. et. al., 2014).

Media and growth Conditions :

The enrichment and isolation of the strains were carried out using Medium 77 (g/ Lit.: K2HP04, 0.5; NH4C1, 1.0; CaC1,.2H20, 0.1; MgS04.7H,0, 0.1; sodium lactate, 5 ; yeast extract, 1.0; FeS0,.7Hz0, 5 ; sodium thioglycolate, 1.0; and ascorbic acid, 1.0). The anaerobic condition was maintained by overlaying the media with sterile paraffin oil. The pure colonies of the strains were isolated from the media after incubation at 65°C. The stock culture were maintained in Medium 77 and preserved at 4°C for further use.

Characterization of the isolates :

I) Morphological Studies and Biochemical Studies :

The thermophilic isolate was identified by presumptive conventional, physiological and biochemical tests. These tests were (according to Bergey's manual) Gram reaction, catalase production, hydrolysis of protein, starch and lipid, and acid production from sugar. The species was reconfirmed in an automated Biomerieux Vitek 2 System (At Nucleus Diagnostic Centre, Kalyan).

II) Optimization of Growth Conditions:

Determination of the Optimum pH, Temperature and Sodium Chloride Concentration:

The optimum pH for growth was determined using phosphate buffer, universal buffer, and Tris –HCI buffer to obtain different pH values in the range of 4.0 to 9.0 pH and was confirmed using pH meter. For finding out optimum temperature, cultures were streaked onto agar plates and incubated at a range of temperatures from 50-80°C. The plates were observed daily up to 5 days.

For NaCl tolerance level, 100 ml of isolation medium prepared in a phosphate buffer at final concentration of 50mM and at a final salt (NaCl) concentration of 0.5%, 1.5%, 3.0%, 4.5%, 6.0% and 7.5%,. 1 ml of culture was added and the flask was incubated at 41°C in an orbital shaker running at 200 rpm. The growth was determined at 3h intervals by measuring the O.D at 550 nm. (For all the experiments anaerobic conditions were maintained as described above).

III) Sample Collection of effluent from industries:

The effluents having high concentration of sulphate from 3 industries viz., Textile, Battery, Paper & pulp; within the city zone of Thane were collected into polyethelene bottles (500ml). The sample was first analysed to find out the concentration of sulphate and sulphide (Turbidometric method, and Iodometric Method respectively, APHA). Effluents were then exposed to TSRP for reduction of sulphate to sulphide.

IV) Biosulphidogenesis:

Sulphate reduction rate (SRR) and sulphide production rate (SPR) for standard sulpahte and for effluent were determined by method described in APHA. The rates were found out in the interval of 2 hrs. till 24 hrs. (All the experiments were done in triplets).

V) Strain identification by 16s rRNA Analysis:

The isolated colony was sequenced for its conserved sequences and analysed for partial 16s rRNA (Sequencing was done at geneOmbio, Pune, Maharashtra).

The predicted 16S rRNA sequences were compared using the program BLAST (ftp://ftp.ncbi.nih.gov/BLAST/executables/LATEST/).

VI) Phylogenetic and Evolutionary Analysis :

Partial 16S rRNA gene sequences representing the 5 most prevalent OTUs (Operational Taxonomic Units) from thermophilic environment (NCBI database) were aligned using CLUSTALW. Phylogenetic and molecular evolutionary analyses were performed using software cladogram.

III. Results and Discussion

The strain isolated from the hot water spring Si was anaerobic, extremely thermophilic bacteria with optimum temperature of 60, pH 5.5 and NaCl tolerance level upto 1.5%. The biochemical properties are shown in Table 1 and 2.

The isolated strain showed complete reduction of standard sulphate in 20 hr with negligible production of sulphide. And assessment of effluent from the industry showed the rate of reduction of complete sulphate in 25, 24, 22 hrs. respectively with negligible production of sulphide, refer table 3.

Table 1: Biochemical Properties of TSRP (According to Bergey's manual)

Characteristics	Strain si
Gram Nature	Gram Negative
Shape	Curved
Motility	Non motile
Temperature°C – Optimum	60
pH – Optimum	5.5
NaCl % - Optimum	1.5
Oxidase	-
Catalase	-
Casein	-
Starch	+
D-Glucose	+
D-Fructose	-
Maltose	-
Mannose	-
Trehalose	+
Sucrose	-
Mannitol	+
Melibiose	-
Lactose	+
Xylose	-
Galactose	+
Nitrate	+
Citrate	+

Key : Growth : + ; No Growth : -

Table 2: Biochemical Tests (By Biomerieux Vitek 2 System)

Biochemical Details																	
2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	d CEL	-	9	BGAL	+
10	H2S	+	11	BNAG	-	12	AGLTp	-	13	d GLU	+	14	GGT	-	15	OFF	+
17	BGLU	+	18	dMAL	-	19	dMAN	+	20	dMNE	-	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	+	34	d TAG	-	35	d TRE	+	36	CIT	+	37	MNT	-	39	5KG	+
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	+	53	IHISa	-	56	CMT	+	57	BGUR	-
58	0129R	-	59	GGAA	-	61	IMLTa	+	62	ELLM	-	64	ILATa	+			

Table 3: Initial and final concentration of sulphate and sulphide before and after the treatment of effluent collected from 3 industries by TSRP strain Si.

Industry / Standard	Sulphate concentration (mg/L)			Sulphate Reduction rate (%)	Sulphide concentration (mg/L)		Increase in Sulphide Production rate (%)
	Initial	Final	Reduction Time (hrs.)		Initial	Final	
Standard Sulphate	500	0	20	100	--	0.5	0.5
Colour and dye Industry	689	0	25	100	15	16.1	1.1
Paper and Pulp	789	0	24	100	20	20.8	0.8
Chemical	869	0	22	100	59	60	1

16s rRNA Analysis :
The sequence of conserved sites by partial sequence analysis gave the following sequences.
>488 16S1
ACGACGGGTAGCCGGCCTGAGAGGGTGGTCGGCCACGCGGGCACTGAGACACGGGCCCGACTCCT
ACGGGAGGCAGCAGGGGGGAATCTTGGGCAATGGGCGAAAGCCTGACCCAGCGACGCCGCGTGG
GGGAAGAAGGCCTTCGGGTCGTAAACCCCTGTTCTGGAGGAAGAACCCAGGGTAGGTGAATAACC
TATCCTGGCTGACGGTACTCCAGGAGAAAGCCACGGCTAACTGCGTGCCAGCAGCCGCGGTAATA
CGCAGGTGGCGAGCGTTGCCCGTAAAGGGTGCGTAGGCGGCCGGACAAGTCATAGGTTAAAGCCC
GGAGCTCAACTCCGGAAAGGCCTATGATACTGTCTGGCTTGAGGGCCGGAGAGGCTGGCGGAATT
CCCGGTGTAGGGGTGAAATCCGTAGATATCGGGAGGAACACCGGTGGGGAAGCCGGCCAGCTGG
ACGGTTCCTGACGCTGAGGCACGAAAGCGTGGGGA

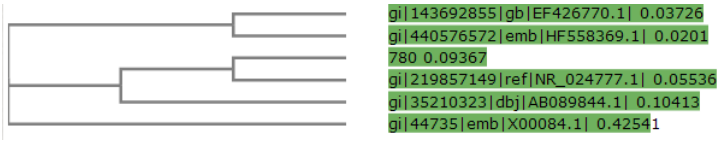
The sequence were aligned with the existing database and it was found that the srtain isolated was of Thermodesulfobacterium commune. The data has been deposited in Genbank with accession number KJ868727. The phylogenetic relationship between thermophilic OTUS showed the following table of similarity index,Table 4.

Table 4 The Score Table : Percent Similarity Index (As per Sequence Similarity)

SeqA	Name	Length	SeqB	Name	Length	Score
1	*Thermodesulfobacterium*	488	2	gi|143692855|gb|EF426770.1|	409	44.5
1	*Thermodesulfobacterium*	488	3	gi|44735|emb|X00084.1|	292	42.47
1	*Thermodesulfobacterium*	488	4	gi|440576572|emb|HF558369.1|	1529	77.66
1	*Thermodesulfobacterium*	488	5	gi|35210323|dbj|AB089844.1|	1500	74.59
1	*Thermodesulfobacterium*	488	6	gi|219857149|ref|NR_024777.1|	1506	80.33

KEY :
Seq 1 *Thermodesulfobacterium commune*
Seq 2 Geobacillus sp. DDS021
Seq 3 Methanococcus vanniellii
Seq 4 Thermus thermophilus
Seq 5 Sulfobacillus thermosulfidooxidans
Seq 6 Thermanaeromonas toyohensis

Phylogram :



IV. Discussion

The present study intended to identify the disimilatory sulphate reduction in thermophilic bacteria. Strain Si showed 100% reduction of sulphate 20 hrs. The analysis of the effluent collected from various industries showed sulphate level above the permissible limit (500 ppm). These effluents were subjected for sulphidogenesis by TSRP strain Si, and 100% reduction rate was found in 25,24,22 hrs. with negligible production of sulphide. It takes more time for the reduction of sulphate in the effluent sample, may be because of presence of other elements like H2S, Cd, Ni, Cu, Cd, Cr, Pb that slow downs the rate of sulphate reduction (Murhekar Gopalkrushna Haribhau,et.al.,2012)

Biochemical tests (Both Manual and automated method) and 16s rRNA analysis confirms that species of reference is of Genus *Thermodesulfobacterium* and species as *commun*e. The evolutionary studies showed 80% similarity index with *Thermanaeromonas toyohensis*.

The most important characteristic of this strain is high potential of sulphate reduction with negligible production of sulphide, thus there is no foul odour and even no precipitation of other metal present in the effluent (Z. Manafi, et.al.,2013). Considering all these aspects *Thermodesulfobacterium commune* (Accession Number KJ868727) will be an effective sulphate reducing thermophilic bacteria.

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