Research Journal of Recent Sciences _____ ISSN 2277-2502
Vol. 3**(ISC-2013),** 203-208 **(2014)** *Res. J. Recent. Sci.*

Biosulphidogenesis and Bioaccumulation of sulphate by moderately Thermophilic, Facultative anaerobic Bacteria *Aeromonashydrophila*isolated from hot Water spring

Patil S. Z., Unnitha. A. R. and Unnikrishnan G.
Department of Biotechnology, Birla College of Arts, Science and Commerce, Kalyan, Maharastra, INDIA

Available online at: www.isca.in, www.isca.me
Received 9th January 2014, revised 23rd February 2014, accepted 28th March 2014

Abstract

A unique, facultative anaerobic, moderately thermophilic Sulphate reducing prokaryote (TSRP) was isolated from Vajreshwari and Ganeshpuri hot Springs of Thane, Maharashtra. The optimum temperature, pH and NaCl concentration for growth of this strain was found to 41°C, 6.5, and 4.5% respectively. The strain Si showed 100% reduction ofstandardsulphatein 11hrs., with no production of sulfide. This result reflects the presence of assimilatory sulfate reduction pathway type I, which reduces sulfate to sulfite and finally to sulfide that is accumulated in the cellfor cysteine biosynthesis. The analysis of the effluent collected from colour and dye industry showed high concentration of sulphate (689ppm).These effluent wassubjected to sulphidogenesis by strain Si and there was complete reduction of sulphate in 12.30 hrs.with no production of sulphide. Phylogenetic analysis of 16s rRNA sequence placed strain Si in gamma subclass of proteobacter, showing highly similarity with other phylogenetic relatives. Thus this isolate is a member of genus Aeromonas and the type strain is hydrophila strain ZHYYZ-1. The result indicated that Aeromonashydrophila strain ZHYYZ-1 has high efficiency of sulphate reduction in much less time with no production of sulfide than previously studied anaerobic bacteria.

Keywords: Biosulphidogenesis, TSRP, Assimilatory Sulphate reduction pathway Type I, Bioaccumulation, 16s rRNA.

Introduction

Sulphates are occur naturally occurring element and are used in the manufacture of chemicals, dyes and fertilizers, in the mining and pulping industries, in sewage treatment and in wood preservation[1].

Sulphates are discharged into the aquatic environment through effluents from industries like mining, smelting, pulp and paper mills. Surface water can also be contaminated by atmospheric sulphur dioxide[2].

Sulphate is one of the least toxic anions. The major physiological effects resulting from ingesting large quantities of Sulphate are catharsis and gastrointestinal irritation. These effects are enhanced when Sulphate is consumed in combination with magnesium[1]. The maximum permissible limit for Sulphate in water is 500ppm (WHO). Thus, biosulphidogenesis (Sulfide or elemental Sulfur generation) is necessary before discharging the Sulphate containing effluent into water body. Since the hot springs contain a wide range of sulphate concentration, thermophile microorganisms are being used for sulphidogenesis nowadays. The reduction of sulphate by this process is environment friendly and economically viable hence they are being widely used in bioremediation process[3].

Thermophilic sulfate-reducing prokaryotes (TSRP) have increasingly attracted interest due to their potential in various biotechnological applications, such as biosulphidogenesis and biohydrometallurgical processes[4].

One of the best applications of TSRP is treating the effluent having sulphateconcentration above the permissible limit. The effluent from Textile, Battery, Paper andpulp, Colour and dye industry contain high amount of sulphate. These runoff ultimately affects the quality of the river or water body beside these industries and effects environment, wildlife as well as human. Hence, it is advisable to treat these effluent for reducing sulphate by TSRP.

The objective of the present study is to enrich, isolate and characterize TSRP from a Vajreshwari and Ganeshpuri hot springs stretching about 7 km in the bed of the River Tansa, Thane, Maharastra. (http:// www.mumbaisuburbs.com / mumbai-tourist / travelvajreshwari.html) and study biosulphidogenesis of industrial effluent containing high amount of sulphate.

Material and Methods

Sampling: Water samples were collected from seven different hot springs (from Vajreshwariand Ganeshpuri, Thane) Mumbai. Surface water samples were taken from the Hot Springs using a grab sampler. Sample was stored in clean polyethylene container with lid. The temperature of the sample was taken with a laboratory thermometer and recorded. All samples were

taken on the same day to prevent discrepancies due to sample date. Water sample used for inorganic analyses was immediately fixed with 3 ml of a 20% (wt/vol) zinc acetate solution for sulfate analysis andwith the same volume of a 90mM zinc acetate solution for determination of the sulfide concentration. In situ spring water used in the test for sulfide production by TSRP was kept in a sterile plastic bottle (1,000ml) [3].

Media and growth Conditions: Medium 77 described by Postgate was used for routine stock maintenance and all enrichment culture studies[5]. Medium 77 contained (g/ Lit.: K2HP04, 0.5; NH_4C1, 1.0; $CaC1_2$,.$2H_20$, 0.1; $MgS0_4$, $7H_2$,0, 0.1; sodium lactate, 5; yeast extract, 1.0; $FeS0_4$,.$7H_20$, 5 ; sodium thioglycolate, 1.0; and ascorbic acid, 1.0). Black colonies were isolated from Medium 77 and 4% (w/v) purified agar. Stock cultures of all strains were prepared from single isolated colonies that proliferated on transfer in Medium 77. All stock cultures were incubated at 50°C.

Characterization and identification of the isolates: Morphological Studies: Morphological properties were investigated by using 18 hour old bacterial cultures. These included the wet mount preparations using light microscope and Gram staining to confirm Gram reaction. Motility was determined by hanging drop method[6].

Biochemical Tests: The thermophilic isolate was identified by presumptive conventional, physiological and biochemical tests. These tests were; Gram reaction, catalase production, hydrolysis of protein, starch and lipid, and acid production from sugar[6]. The species was reconfirmed in an automated Biomerieux Vitek 2 System (At Nucleus Diagnostic Centre, Kalyan).

Optimization of Growth Conditions: Determination of the Optimum pH: The optimum pH for growth was determined by 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[6].

Determination of the Optimum Temperature: Cultures were streaked onto agar plates and incubated at a range of temperatures from 20-60°C. The plates were observed daily up to 5 days[7].

Growth at Different Sodium Chloride Concentration: The experiments were carried out containing 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[5].

Sample Collection of effluent from industries: The effluent from colour and dye industry wasselected as sample for biosulphidogenesis. The sites for sample collection were within the city zone of Thane. Samples were collected in polyethylene bottles. The sample was first analysed to find out the concentration of sulphate (Turbidometric method, APHA). Effluent were then exposed to TSRP for reduction of sulphate to sulphide.

Biosulphidogenesis: Sulfate reduction rate (SRR): Measurement of the reduction of sulphate into sulphide was performed method described in APHA. The rate of sulphate reduction was determined from points taken during a 10 hr time course. All assays were done in triplet.

Sulfide production rate: Dissolved sulfide concentrations were estimated by using Iodometric method (APHA) after in situ fixation with a zinc acetate solution. Concentration of sulfide was detemined both before and after process of sulphate reduction to estimate actual amount produced by biosulphidogenesis. All assays were done in triplet.

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 gene Ombio, Pune, Maharashtra).
The predicted 16S rRNA sequences from this study were compared with 16S rRNA sequences in database available in ribosomal database project (release 8.1;http:// rdp8. cme.msu.edu.). Comparisons were made using the program BLASt (ftp://ftp.ncbi.nih.gov/ BLAST/ executables/LATEST/)[4].

Phylogenetic and Evolutionary Analysis: For cladogram construction, partial 16S rRNA gene sequences representing the 15 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.

Results and Discussion

Characterization of in situ sulphidogenesis: Microbial sulphate reduction at high temperatures was studied using samples collected from several sites in Vajreshwariand Ganeshpuri hot springs. Enrichment cultures were initiated with Medium 77. After incubation of 24 hrs.dense black coloured colonies were transferred to fresh media for identification.

Cellular properties: Cells appeared as very tiny straight rods. Motility was not observed. Exponential phase cells stained Gram-negative and lacked catalase. The optimum peak concentration for growth was at 4.5% NaCl. Other biochemical properties (As per Bergey's manual) are described in table 1. Species was again confirmed by using automated system of Biomerieux System (Tested at Nucleus diagnostic Centre, Kalyan) stated as in table-2.

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

Characteristics	*Strain si*
Gram Nature	Gram Negative
Shape	Curved
Motility	M
Temperature°C – Optimum	41
pH - Optimum	6.5
NaCl % - Optimum	4.5
Oxidase	-
Catalase	-
Casein	+
Starch	+
D-Glucose	+
D-Fructose	+
Maltose	+
Mannose	-
Trehalose	-
Cellobiose	-
Sucrose	-
Mannitol	-
Melibiose	+
Lactose	+
Arabinose	-
Xylose	-
Galactose	+
Nitrate	-
Citrate	-

Key: Growth : + ; No Growth : - ; M : Motile

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	-			

Growth and metabolic properties: Long lag period was not observed and adoubling time of less than 4 hr was observed during the exponential growth phase, figure 1.
Bacterium had an optimal growth temperature near 41^{o}C; it grew below 65° C and above 37°C, figure2.

Themicrobe showed growth in between pH range of 6.0 to 8.0 in Medium 77 with an optimal pH near 6.5.and optimum growth was found at NaCl concentration of 4.5%.

Standard sulphate Reduction: 100 % reduction was observed for a standard sulphate from500 mg/L (ppm) to 0 mg/L (ppm) in 11 hrs without the production of sulfide, table 3.

Effluent treatment: The effluent from colour and dye industry contained high concentration of sulphate well above the permissible limit (500 ppm). The time taken by TSRP for complete reduction of sulphate was found to be 12.30hrs.with no production of sulphide, table3.

16s rRNAAnalysis : The sequence of conserved sites by partial sequence analysis gave the following sequences.
TCGCAATTGGCGGGCGGTCTACACATGCAAGTCGAGC
GGCAGCGGGGAAAGTAGCTTGCTACTTTTGCCGGCGA
GCGGCGGACGGGTGAGTAATGCCTGGGAAATTGCCCA
GTCGAGGGGGATAACAGTTGGAAACGACTGCTAATAC
CGCATACGCCCTACGGGGGAAAGCAGGGGACCTTCGG
GCCTTGCGCGATTGGATATGCCCAGGTGGGATTAGCTA
GTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCT
AGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTG
AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGCACAATGGGGGAAACCCTGATGCAGCCAT
GCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCA
CTTTCAGCGAGGAGGAAAGGTTGATGCCTAATACGTA
TCAACTGTGACGTTACTCGCAGAAGAAGCACCGGCTA
ACTCCGTG

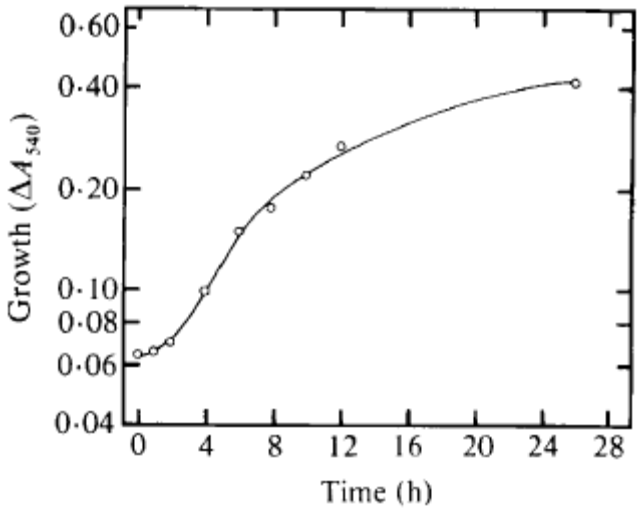
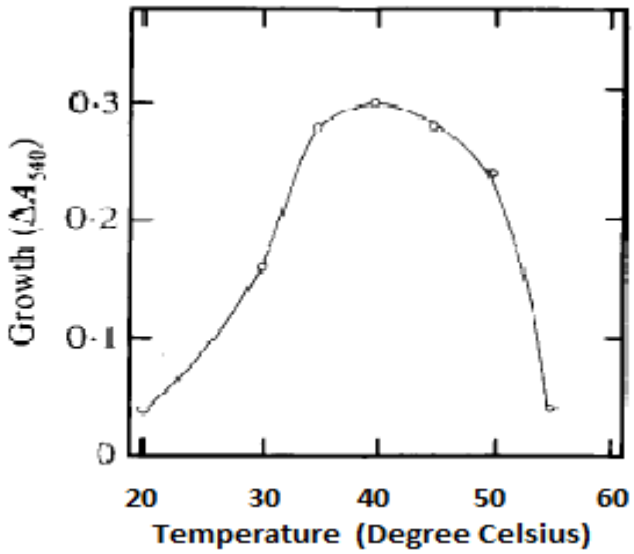


Figure-1
Growth curve of *Strain Si* cultured on sulphate in modified Medium 77



20 30 40 50 60
Temperature (^{O}C)

Figure-2
Optimum temperature and growth yield of *Strain Si*. The organism was cultured in Medium 77 at the temperatures indicated

The sequence was aligned for comparison with the existing databases by using in silico tool BLAST. The isolate was found to be of genus *Aeromonas* and type strain was found to be *hydrophila* strain ZHYYZ-1.

The phylogenetic and evolutionary analysis of the operational taxonomic units isolated from thermophilic environment was analysed by CLUSTALW, figure 3 and it showed the following relationship between the different species. The score table showed the % similarity of these strain and all other OTUs, Table 4.

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)		Sulphide Production rate (%)
	Initial	Final	Reduction Time (hrs.)		Initial	Final	
Standard Sulphate	500	0	11	100	--	--	0
Colour and dye Industry	689	0	12.30	100	12	12	0

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

Accession number of Query Organism - >gi|290792568|gb|GU563992.1| Aeromonashydrophila strain ZHYYZ-1	
Accession Number of OTUs	Score
>gi|44194255|gb|AY538658.1| *Aeromonashydrophila strain HY03*	96.69
>gi|238814979|gb|FJ972531.1| *Pseudomonas aeruginosa*	85.06
>gi|82941283|dbj|AB242868.1| *Marinomonasostreistagni*	84.86
>gi|188219225|emb|AM999769.1| *Thermusaquaticus*	76.60
>gi|143692855|gb|EF426770.1| *Geobacillus sp. DDS021*	76.53
>gi|219857149|ref|NR_024777.1|*Thermanaeromonas toyohensis strain ToBE*	76.16
>gi|51036225|dbj|AB186359.1| *Clostridium clariflavum*	75.68
>gi|440576572|emb|HF558369.1| *Thermus thermophiles*	75.68
>gi|540352423|emb|HG380021.1| *Streptococcus thermophiles*	74.47
>gi|576620|gb|L37731.1|VIBRR16SAG *Vibrio anguillarum*	73.75
>gi|378405442|gb|JQ346745.1| *Thermodesulfobacterium commune YSRA-1*	73.35
>gi|35210323|dbj|AB089844.1| *Sulfobacillusthermosulfidooxidans*	73.08
>gi|61653285|dbj|D28576.2| *Sphingomonasmali*	63.33
>gi|44735|emb|X00084.1| *Methanococcusvanniellii*	49.66

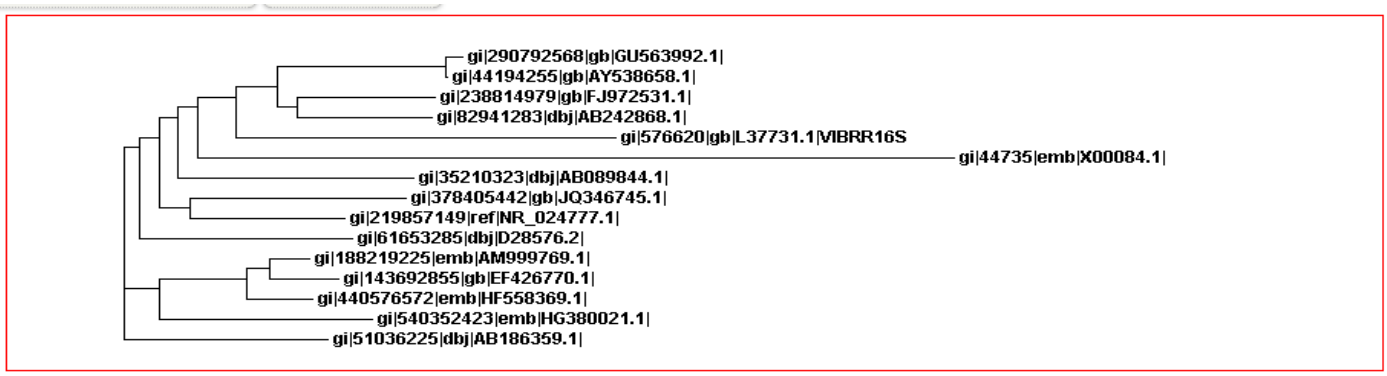


Figure-3
Cladogram

Discussion: The present study intend to identify the assimilatory sulphate reduction in thermophilic bacteria[8]. Bacterial sulphate reduction at high temperatures appears wide spread in Vajreshwari and Ganeshpuri hot springs and was associated with at leastone species. Thermophilic sulphate-reducing bacteria were found in hot water spring where sulphate content and organic matter content are more. Biosulphidogenesis appeared most active in the sulphate-depleted thermal ecosystem, where microbial sulfate reduction occurred.

The isolated strain *Si* was found to be facultative anaerobic, moderate thermophilic, Gram negative rods. Optimum temperature for growth was 41°C. pH range 6.0-8.0. Growth inhibited by 4.5% NaC1.

Strain *Si* showed complete reduction of standard sulphate (500ppm to 0 ppm) in 11hr with no production ofsulphide production. Experiments by J. Suschka and L. Przywara(2006) showedthat 90 % sulphate reduction was achieved after 60hrs. by using *Desulfovibrio or Desulfobacterium* with sulfide production as high as 150 mg/L. This showed that as compared with anaerobic sulphate reduction, the reduction rate by aerobic TSRP is faster and more effective as there is no production of sulfide. The reason behind this is that the facultative aerobes require the sulphide for synthesis of other biomolecule like cysteine (Biocyc database). Thus sulphate is reduced to sulfide first and then it is consumed for their nutrient requirement. This indicates that the rate of sulphate reduction seems to be high in this strain as compared to anaerobic organism (where dissimilatory reduction is present)[9].

The analysis of the effluent collected from Colour and dye industry showed sulphate level above the permissible limit (500 ppm)[10]. These effluent was subjected for sulphidogenesis by TSRP strain *Si*, and the reduction rate was found to be 100% in 12.30 hrs.withno production of sulphide. It takes more time for reduction of the effluent sample,may be because of presence of other elements like H2S, Cd, Ni, Cu, Cd, Cr, Pb (Reis MA, Almeida JS, Lemos PC, Carrondo MJ., 1992 and Aili Tan, Kaixuan Tan, Zhengji Yi 2004) that slow downs the rate of sulphate reduction.

Biochemical tests (Both Manual and automated method) and 16s rRNA analysis confirms that species of reference is of Genus *Aeromonas* and species as *hydrophila.* strain ZHYYZ-1. The phylogenetic analysis and percent similarity indexnshowed that *A. hydrophila*strainZHYYZ-1have 96.69% similarity with its closely related species of *A. hydrophila strain HYO.*

The score table showed that sequences in study has 85.06%, 84.86% similarity with *Pseudomonas aeruginosa*and *Marinomonasostreistagni*respectively. The evolutionary tree showed relationship between all the 15 species. It was found out that *A. hydrophila*strain ZHYYZ-1, *A. hydrophila*HYO strain,*Pseudomonasaeruginosa*and *Marinomonasostreistagni*are evolved from common ancesters.

The study showed that *A. hydrophila* strain ZHYYZ-1 being a facultative anaerobe, does not require any specific bioreactor as thtat are needed for anaerobic organism. The most important factor is that have high potential of sulphate reduction with no production of sulphide, thus there is no foul odour and even no precipitation of other metal present in the effluent. Considering all these aspects *A. hydrophila*strainZHYYZ-1 will be an ideal candidate for biosulphidogenesis and will be best way for the treatment of industrial effluent containing high level of sulphate

Conclusion

The isolated moderarely thermophilic organism *Aeromonashydrophila.* strain ZHYYZ-1 was Gram negative rods with facultative anaerobic strain which has higher sulphate reduction rate than anaerobic TSRP with no production of sulfide.

References

1. Cloetete., Gerber a. and Maritz l. V., A first order inventory of water use and effluent production by SA industrial, mining and electricity generation sectors, Arcusgibb (pty) ltd, *water resource division (water – north)*, pretoria, wrc report no. 1547/1/10 **(2010)**

2. KrishnanandanVivek, Srikantaswamy S, Assessment of impacts by Industries on sediments of Kabini river around Nanjangud Industrial area, Karnataka, India, *International Journal of Scientific and Engineering Research*, **4(11) (2013)**

3. Vemula M., Ambavaram V., Kalluru G. and Gajulapalle M., Tollamadugu N. An Overview on Research Trends in Remediation of Chromium, *Research Journal of Recent Sciences***, 2(1),** 71-83, January **(2013)**

4. Anna H. Kaksonen, Jason J. Plumb,Wendy J. Robertson, Stefan Spring, Peter Schumann, Peter D. Franzmann and Jaakko A. Puhakka, Novel Thermophilic Sulfate-Reducing Bacteria From A Geothermally Active Underground Mine In Japan. Appl *Environ Microbiol*., May, **72(5),** 3759–3762 **(2006)**

5. Carine Audiffrin, Jean-Luc Cayol, Catherine Joulian, Laurence Casalot, Pierre Thomas, Jean-Louis Garcia and Bernard Ollivier, *Desulfonauticussubmarinus* gen. nov., sp. nov., a novel sulfate-reducing bacterium isolated from a deep-sea hydrothermal vent, *International Journal Of Systematic and Evolutionary Microbiology*, **(5),** 1585-1590 **(2003)**

6. Francis Amala Rejula and Masilamai Dhinakaran, Removal of Zinc (II) by Non Living Biomass of Agaricus Bisporus, *Research Journal of Recent Sciences*, **1(9),** 13-17 **(2012)**

7. Elisa Bayraktarov, Roy E. Price, Timothy G. Ferdelman and Kai Finster, The pH and pCO_2 dependence of sulfate reduction in shallow-sea hydrothermal CO_2 – venting sediments (Milos Island, Greece*), Front. In Micro* **(2013)**

8. Mehar Fatma, M. Iqbal R., Khan Asim Massod and nafees A. Khan, Coordinate changes in assimilatory Sulphate reduction are correlated to salt tolerance : Involvemetn of Phytohormones, Annual review and Research in biology, **3(3) (2013)**

9. Z. Manafi1, M. Hashemi1, H. Abdollahi,Gregory. J. Olson, Bio-corrosion of water pipeline by sulphate-reducing bacteria in a mining environment, *Research Journal of Recent Sciences,* **12(46),** 6504-6516 (**2013)**

10. Murhekar Gopalkrushna Haribhau, Trace Metals Contamination of Surface Water Samples in and Around Akot City in Maharashtra, India, *Research Journal of Recent Sciences,* **1(7),** 5-9 **(2012)**