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# An Investigation Into The Mechanisms Underlying Enhanced Biosulphidogenesis In *Bacillus* Spp.

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***Abstract:*** *Anthropogenic activities like mining, processes of metallurgy and other chemical industries lead to the discharge of a high amount of sulphate into the environment that causes serious problems to human health. This paper illustrates the employment of thermophilic sulphate reducing bacteria for biosulphidogenesis. Two different species have been isolated from hot water spring of Vajreshwari and Ganeshpuri,Thane, Maharashtra, INDIA.The mechanism involved in biosulphidogenesis includes production of specific protein as well as liberation of some extracellular polymeric compound (EPS) e.g. proteins, carbohydrate, acids etc. that are produced during the microbial cell metabolism. These compounds plays an important role in the faster reduction of sulphate and decrease in production rate of sulphide.The isolate was found to be of genus Bacillusand type strain was found to be subtilis Zankar and licheniformis Sonali. The strain sequence were deposited in NCBI database with accession number KJ939324 and KJ939325 respectively. The result highlights the potential use of these organism in biosulphidogenesis.*

***Keywords:*** *Sulphate reducing bacteria, Protein Expression, Extracellular polymeric Substances, FTIR.*

## I. Introduction

Thermophilic Bacteria are ubiquitous and highly diverse. They can survive in all sorts of inhospitable environments. Studies in the last two decades have revealed that 99% of bacteria present in the environment are still unexplored or overlooked in laboratory cultivation and hence remain obscure for their ecological functions and unexploited for biotechnological applications.

One of the best application of thermophiles is reduction of sulphate. Sulphate Reducing Bacteria (SRB) are currently subjected to extensive genomic studies, to understand their basic biochemical mechanisms, which would help in the development of novel techniques for the analyses of their role in environment. SRB uses different sort of mechanism for reduction of sulphate,which includes production of specific enzymes, extracellular polymeric substances (EPS), etc.

The expression of metal-binding proteins or peptides in microorganisms in order to enhance sulphate accumulation and/or tolerance has great potential. Several different peptides and proteins have been explored. The mechanisms involved in bacterial metal resistance result from either the active efflux pumping of the toxic metal out of the cell, or the enzymatic detoxification (generally redox chemistry) converting a toxic ion into a less toxic or less available metal ion.

Another mechanism is EPS,which are a complex high-molecular-weight mixture of polymers excreted by microorganisms, produced from cell lysis and adsorbed organic matter from wastewater. Their characteristics (e.g., adsorption abilities, biodegradability and hydrophilicity/hydrophobicity) and the contents of the main components (e.g., carbohydrates, proteins, humic substances and nucleic acids) in EPS are found to be crucially affecting the properties of microbial aggregates, such as mass transfer, surface characteristics, adsorption ability, stability, the formation of microbial aggregates etc.

So the objectives of this study was to isolate thermophilic sulpahte reducing bacteria, identify them and to investigate the mechanism of sulphate reduction present in the isolated species.

## II. Methods And Materials

**Sampling:**

Water samples were collected from seven different hot springs (from Vajreshwari & Ganeshpuri, Thane) Mumbai. Surface water samples were taken from the Hot Springs using a grab sampler. A 500-ml plastic cup attached to a 2-m pole was dipped into the water twice to rinse it. The sample was then transferred to a clean, new, polyethylene container with a snap-on 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. Samples were kept cool during transport to the laboratory and processed within 12 h of collection **(Vieille C.,**1996). Spring water used for inorganic analyses was immediately fixed with 3 ml of a 20% (wt/vol) zinc acetate solution for sulfate analysis &with 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).

**Media & growth Conditions**

Medium D described by Postgate (1969) was used for routine stock maintenance and all enrichment culture studies. Medium D contained (g/ Lit.: Na Pyruvate - 3.5g , MgCl2.6H2O - 1.6g , NH4Cl - 1g , Yeast Extract - 1g ,KH2PO4 - 0.5g , CaCl2.2H2O - 0.1g , FeSO4.7H2O - 0.04g , pH 7.5 ± 0.2 (25˚C). Black colonies were isolated from anaerobic roll tubes (Hungate, 1969) containing Medium D and 4% (w/v) purified agar. Stock cultures of all strains were prepared from single isolated colonies that proliferated on transfer in Medium D. All stock cultures were incubated at 50°C. Cultures were routinely checked for contamination by the detection of growth in the complex medium, TYEG, described for growth of saccharolytic thermophiles (Zeikus*et al*., 1979).

**Characterization and identification of the isolates :**

**I) Morphological Studies :**

Morphological properties were investigated by using 18 hour old bacterial cultures. These included the wet mount preparations using light microscope & Gram staining to confirm Gram reaction. Motility was determined by hanging drop method.

**II) Biochemical Tests :**

Two thermophilic isolate was identified by the use of conventional methods for the presumptive identification of physiological and biochemical tests. These tests were; Gram reaction, catalase production, hydrolysis of protein, starch and lipid, and acid production from sugar **(Campos,** *et al***,**1995). The species was reconfirmed by using automated BiomerieuxVitek 2 System (Nucleus Diagnostic Centre, Kalyan).

**Biosulphidogenesis:**

**a) Sulfate reduction rate (SRR) :**

Measurement of the reduction of sulphate into sulphide was performed via the exact procedures detailed by Ingvorsen*et al.* (1981) for determination of bacterial sulphate reduction in anoxic water and sediment samples. The rate of sulphate reduction was determined from points taken during a 10 hr time course. (All assays were done in triplet.)

**b) Sulfide production rate :**

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

**Mechanisms involved in biosulphidogenesis :**

**a) Isolation of Proteins :**

The bacterial cell of both the strains were grown at 45 C for 24 hrs. and the cells were harvested by centrifugation at 13,000 rpm for 10 mins. Later the cell pellet was washed with phosphate buffer (pH 7.0) to remove the traces of remaining media and again centrifuged at 10,000 rpm for 10 mins. The supernatant was discarded. The cell pellet obtained was mixed with 1ml of 2X sample buffer (0.5% SDS, 25% beta mercaptoethanol, 0.03% bromophenol blue, 2.5% Glycerol, 15mM trisHCl (pH 6.8)). The samples were vortexed and incubated in a boiling water bath for half an hour. The samples were used directly for SDS-PAGE analysis (Maiti et al., 2009). The same method of isolation was performed with and without the treatment of sulphate (control and Test).

The most probable protein matching according to molecular weight was carried out by insilico method using by http://web.expasy.orgDatabase.

**b) Isolation of EPS :**

Six day-culture broths (250ml nutrient broth containing test organisms incubated at 50˚C at 80rpm) were centrifuged (6000 ×g, 30mins, 4˚C). the EPS were isolated either (i) from bacterial pellets or (ii) from supernatants.

i. The cell pellets from the culture were suspended in 10ml (20g /liter) NaClin water and were centrifuged (6000 ×g, 30mins, 4˚C). the rsulting pellets were suspended in 10ml (10g /liter) NaCl in water and were dialyzed against water for 48 hours. The dialyzed samples were centrifuged (6000 ×g, 30mins, 4˚C), and stored at 4˚C.
ii. The supernatants were filtered through Whatman Filter paper no 40. After the addition of cold ethanol (ethanol/filtrate ratio 2:1), the solutions were kept overnight at 4˚C. The EPS precipitates were recovered by centrifugation (6000 ×g, 30mins, 4˚C).

The pellets were suspended in water and stored at 4˚C. The protein contents of EPS was determined by Barfoeds method and total neutral- carbohydrate content was determined by phenol-sulphuric acid method and were confirmed by FTIR analysis.The same method of isolation was performed with and without the treatment of sulphate (control and Test) (Fabienne Francois, *et. al*.2011).

**FTIR Analysis for EPS**

Samples for FTIR can be prepared in a number of ways. For liquid samples, the easiest is to place one drop of sample between two plates of KBr (salt). Salt is transparent to infrared light. The drop forms a thin film between the Plates which was analysed by FTIR. The plates were scanned from 400 to 4000 wavenumber. The functional group from each spectra was determined using co-relation chart.

**Strain identification:**

It was done by 16s rRNA Analysis and the isolated colonies were sequenced for its conserved sequences and analysed for partial 16s rRNA by geneOmbio, Pune, Maharashtra.

The predicted 16S rRNA sequences from this study were compared with 16S rRNA sequences in a BLASTable database constructed from sequences downloaded from the 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/) and a FASTA-formatted file containing the predicted 16S rRNA sequences.

## III. Results

**Characterization of in situ sulphidogenesis:**

Microbial sulphate reduction at high temperatures was studied at several sites in Vajreshwari & Ganeshpuri hot springs. Enrichment cultures were initiated with Medium D. After incubation of all enrichments for 6 d at *in situ* temperature, the cultures formed a dense black precipitate. Repeated transfer of enrichments revealed small rod-shaped sulphate reducing bacteria in all cultures. All strains appeared morphologically identical with one being mucoid colony (SZP 1) and one with smooth colony (SZP 2) when cultured on Medium D at 41°C. Stock cultures of both strainwere maintained on Medium D and transferred monthly.

**Cellular properties:**

Cells appeared as very tiny straight rods. Motility was not observed. Exponential phase cells stained Gram-positive. Other biochemical properties (As per Berget,s manual)are described in Table 1. Species was again confirmed by using automated system of Biomerieux System (Done at Nucleus diagnostic Centre, Kalyan) stated as in Table 2 and 3.

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

| Characteristics | *Strain SZP 1* | *Strain SZP 2* |
|---|---|---|
| Gram Nature | Positive | Positive |
| Shape | Bacillus | Bacillus |
| Motility | M | M |
| Temperature°C– Optimum | 41 | 45 |
| pH - Optimum | 6.5 | 7.0 |
| NaCl % - Optimum | 1.5 | 1.4 |
| Oxidase | + | + |
| Catalase | + | + |
| Casein | + | + |
| Starch | + | + |
| D-Glucose | + | + |
| D-Fructose | + | - |
| Maltose | - | + |
| Mannose | - | - |
| Trehalose | - | - |
| Cellobiose | - | - |
| Sucrose | - | - |
| Melibiose | + | + |
| Lactose | + | + |
| Xylose | - | + |
| Galactose | - | - |
| Nitrate | - | - |
| Citrate | + | + |

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

**Table 2: Biochemical Tests ( ByBiomerieuxVitek 2 System) For SZP 1**

| Biochemical Details | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | AMY | + | 4 | PIPLC | + | 5 | dXYL | - | 8 | ADH1 | + | 9 | BGAL | - | 11 | AGLU | + |
| 13 | APPA | - | 14 | CDEX | - | 15 | AspA | + | 16 | BGAR | - | 17 | AMAN | - | 19 | PHOS | + |
| 20 | LeuA | - | 23 | ProA | - | 24 | BGURr | + | 25 | AGAL | - | 26 | PyrA | - | 27 | BGUR | + |
| 28 | AlaA | + | 29 | TyrA | + | 30 | dSOR | - | 31 | URE | + | 32 | POLYB | + | 37 | dGAL | - |
| 38 | dRIB | - | 39 | ILATk | + | 42 | LAC | + | 44 | NAG | + | 45 | dMAL | - | 46 | BACI | - |
| 47 | NOVO | - | 50 | NC6.5 | + | 52 | dMAN | - | 53 | dMNE | + | 54 | MBdG | + | 56 | PUL | - |
| 57 | dRAF | - | 58 | 0129R | - | 59 | SAL | + | 60 | SAC | - | 62 | dNTRE | - | 63 | ADH2S | + |
| 64 | OPTO | - | | | | | | | | | | | | | | | |

**Table 3: Biochemical Tests ( ByBiomerieuxVitek 2 System) For SZP 2**

| Biochemical Details | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | AMY | -- | 4 | PIPLC | + | 5 | dXYL | - | 8 | ADH1 | - | 9 | BGAL | + | 11 | AGLU | + |
| 13 | APPA | + | 14 | CDEX | + | 15 | AspA | - | 16 | BGAR | + | 17 | AMAN | + | 19 | PHOS | - |
| 20 | LeuA | - | 23 | ProA | - | 24 | BGURr | - | 25 | AGAL | - | 26 | PyrA | - | 27 | BGUR | - |
| 28 | AlaA | - | 29 | TyrA | - | 30 | dSOR | - | 31 | URE | + | 32 | POLYB | - | 37 | dGAL | - |
| 38 | dRIB | - | 39 | ILATk | + | 42 | LAC | - | 44 | NAG | + | 45 | dMAL | + | 46 | BACI | + |
| 47 | NOVO | - | 50 | NC6.5 | - | 52 | dMAN | - | 53 | dMNE | + | 54 | MBdG | - | 56 | PUL | - |
| 57 | dRAF | - | 58 | 0129R | - | 59 | SAL | - | 60 | SAC | - | 62 | dNTRE | + | 63 | ADH2S | + |
| 64 | OPTO | - | | | | | | | | | | | | | | | |

**Biosulphidogenesis:**

The MIC for both the strains were found to be above 1000 ppm. The reduction rate for standard Sulphate was found to be 100% in 14 hrs. for SZP 1 and 90% for SZP 2 in 14 hrs. with no sulfide production, Table 4.

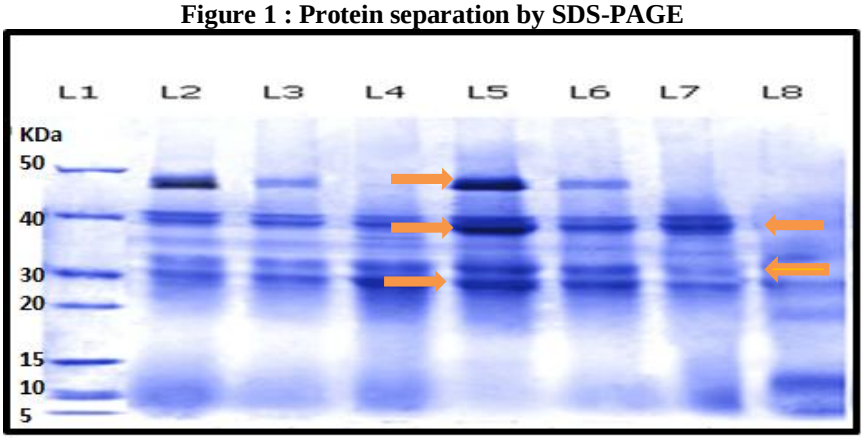
**Table 4: Initial & final concentration of sulphate&sulphide before & after the treatment**

| Strain | Sulphate concentration (mg/L) | | | Sulphate Reduction rate (%) | Sulphide concentration (mg/L) | | Sulphide Production rate (%) |
|---|---|---|---|---|---|---|---|
| | Initial | Final | Reduction Time ( hrs.) | | Initial | Final | |
| SZP 1 | 1000 | 0 | 14 | 100 | -- | -- | 0 |
| SZP 2 | 1000 | 900 | 14 | 90 | -- | -- | 0 |

**Protein Isolation ;**

The gel showed that in presence of sulphate some of the protein/enzyme for both the strains are over expressed. These protein were identifiedby insilico method and most probable protein mathching with the observed results are shown in figure.

**Figure 1 : Protein separation by SDS-PAGE**




**Key: Lane 1 :** Protein Marker, Lane 6: *Bacillus subtilis Zankar* Untreated, Lane 5 *Bacillus subtilis Zankar* treated, Lane 7 : *Bacillus licheniformis Sonali* treated, Lane 8: *Bacillus licheniformis Sonali* untreated

According to molecular weight found, the following protein can be probably expressed during Biosulphidogenesis.

**Bacillus subtilis :**

1) Cytochrome c-oxidoreductase (28,162 Kda)
2) sulphatepermease (36,836 kDa))
3) sulfate adenyltransferase (42884 KDa)

**Bacillus licheniformis :** adenylylsulfate kinase (22456 KDa)

**EPS Production:**

The EPS was extracted from both the organisms and colorimetric quantification of the proteins and carbohydrates was performed. The results indicated the high amount of both proteins and carbohydrate (Table 5) in case of test which was further confirmed by FTIR.

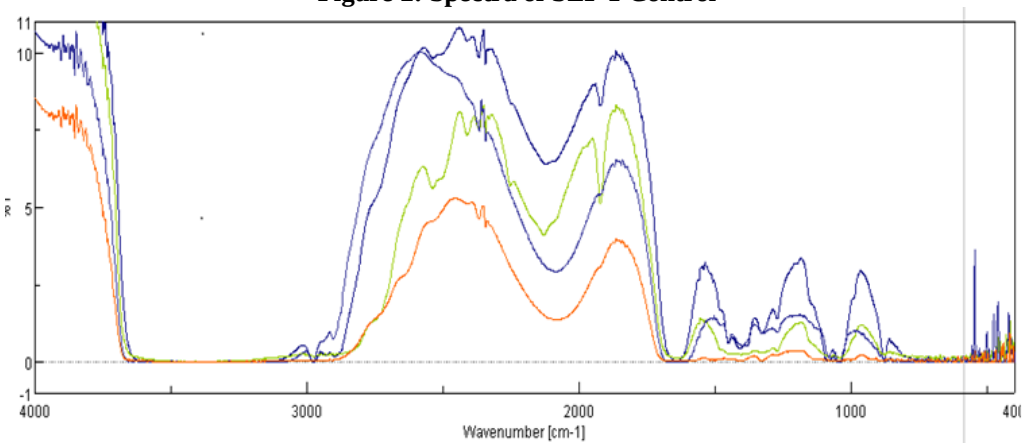
**Table 5: Amount of carbohydrate and protein present in EPS for Control and Test (in treated with sulphate) for strain SZP 1 and SZP 2.**

| Strain | Carbohydrate Content ppm | | Protein Content ppm | |
|---|---|---|---|---|
| | Control | Test | Control | Test |
| SZP 1 | 80 | 89 | 56 | 93 |
| SZP 2 | 68 | 75 | 79 | 84 |

**Fourier Transform – Infrared Spectroscopy Analysis:**

The characterization of EPS by (FT-IR) spectroscopy showed the typical absorption band for carboxylic acid (a broad O—H elongation band in the range 2100 – 3500 $cm^{-1}$) as well as bands for aldehydes and ketones ( a broad H—C=O, C—H stretch in the range 1500-2000$cm^{-1}$) and a band for 1˚ amines (a N—H stretch in the range 750-800 $cm^{-1}$). Thus, the relative measure of intensities confirmed the presence of proteins and carbohydrate in agreement with the colorimetric quantification.

**Figure 2: Spectra of SZP 1 Control**




**Key:** Orange Line: SZP 1 Control ; Blue Line – SZP 1 Treted ; Green line - SZP 2 Control ; Purple Line – SZP 2 Treated

**16s rRNAAnalysis :**

The partial sequence analysis of conserved sites of both the strain gave the following sequences.

**SZP 1**

TTGGCCCCTGCTCTTATCCTTGCCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGG
CGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGC
TAATACCGGATGCTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTT
ACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACAAGGCAACGATGCGTAG
CCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTAGGGAATCTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGTTTT
CGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGG
TACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT

**SZP 2**

GGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATA
CCGGATGCTTGATTGAACCGCATGGTTCAATCATAAAAGGTGGCTTTTAGCTACCACTTGCAGATG
GACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACC
TGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAG
GGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGA
TCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCT
AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTC
CGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCA
ACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGT

AGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTTGTAAC
TGACGTTGAAGGCGGGGCTAAAGGGA

The sequences were aligned forcomparision with the existing databases by using in silico tool BLAST. The isolate was found to be of genus *Bacillus*and type strain was found to be *subtilisZankar and licheniformisSonali*. The strain sequence were deposited in NCBI database with accession number KJ939324 and KJ939325 respectively.

## IV. Discussion

The present study extends the known niche for bacterial assimilatory sulphate reduction in nature to extreme (> 60°C) thermal environments. Bacterial sulphatereduction at high temperatures appears widespread in Vajreshwari&Ganeshpuri hot springs and was associated with one genus with two different colony characteristics. Biochemical test ( Done by Manual and automated method) and also 16s rRNA analysis showed that species found to be of Genus Bacillus& species *as subtilis Zankar and licheniformis Sonali.Both* the strains showed tolerance level of sulphateupto 1000 ppm. Thermophilicsulphate-reducing bacteria were found in environments where organic matter was being vigorously decomposed. Biosulphidogenesis appeared most active in the sulphate-depleted thermal ecosystem, where microbial sulfate reduction occurred.

Both strains showed 100 % and 90% reduction of standard sulphate in 14hr with no production of sulphide production. Experiments by J. Suschka and L. Przywara shows that 90 % sulphate reduction was achieved after 60hrs. by using Desulfovibrio or Desulfobacterium with sulfide production as high as 150 mg/L.

The specified proteins were expressed during the biosulphidogenesis showed that various operons are responsible forthis process.The chemical analysis of EPS and the FT-IR spectroscopy analysis confirmed the presence of proteins and carbohydrates. Further study needs to be performed for identification and characterization of the same and for the use of these organisms for bioremediation of heavy metals.As, the bioremediation of inorganic material is important for health of the people & for monitoring environment, in situ sulphidogenesis by bothBacillus species is one of the best ways for the treatment of industrial effluent containing high levels of sulphate.

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