The Mechanisms Underlying Enhanced Bioremediation in *Thiobacillus* spp

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**Abstract**

Anthropogenic activities like mining, processes of metallurgy and other chemical industries lead to the discharge of high amount of heavy metals into the environment that causes serious problems to human health. This paper aims to employ isolation of thermophilic organisms having capability of bioremediation. Two different *Thiobacillus* species have been isolated from hot water spring of Vajreshwari and Ganeshpuri, Thane, Maharashtra, INDIA. The mechanism involved in bioremediation 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 play an important role in the faster reduction of heavy metals. The isolate was found to be of different species and strain, i.e. *Acidithiobacillus ferrooxidans* POPATNP, *Thiobacillus acidophilus* ABHAY. The sequences of the strain were deposited in NCBI database with accession number KM527210 and KM527215 respectively. The result highlights the potential use of these organism in bioremediation. Thermophilic bacteria are regarded as attractive production organisms for cost-efficient conversion of renewable resources to green chemicals, but their genetic accessibility is a major bottleneck in developing them into versatile platform organisms.

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**1. Introduction**

Given the immense risk posed by widespread environmental pollution by effluents containing heavy metals, novel methods of decontamination and clean-up is required before its discharge (Cloete et al., 2010). Owing to the relatively high cost and the non-specificity of conventional techniques, bioremediation is a promising alternative technology for pollutant clean-up (Kaksonen et al., 2006).

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 unexplored for biotechnological applications.

It was recognized that in situ bioremediation by thermophiles would be faster as compared to mesophiles thus in this model, hot springs serves as a good source for thermophilic microorganisms having novel properties like heavy metal tolerance, thermostability and thermoactivity (Patil et al., 2014). These species uses different sort of mechanism for reduction of heavy metals, 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 heavy metals 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 (Vemula et al., 2010).

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.

2. Objectives of the Research

In situ bioremediation is playing an important role in pollution control. To achieve this objective new greener technology has to be investigated so as to clean up environment in economically viable and simple method. The isolation of microorganisms having capability to bioremediate the heavy metal and to study the mechanism involved in this process will provide a great help to the industrial world for controlling pollution. The objectives of this study was to isolate thermophilic bacteria, identify them and to investigate the mechanisms of heavy metal reduction present in the isolated species.

3. Materials and Methods

3.1 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 et al., 1996).

3.2 Media & growth Conditions

Bacillus Medium described by Postgate (1969) was used for routine stock maintenance and all enrichment culture studies. Bacillus Medium contained (g/ Lit.): Soluble starch – 30.0 g, Agar – 20.0 g, Peptone – 5.0 g, Yeast Extract – 5.0 g, Distilled Water – 1000 ml, pH 7.5 ± 0.2 (45°C). Colonies were isolated from anaerobic roll tubes (Hungate et al., 1969) containing Medium and 4% (w/v) purified agar. Stock cultures of all strains were prepared from single isolated colonies that proliferated on transfer in Media. All stock cultures were incubated at 50°C. Cultures were routinely checked for contamination (Zeikus et al., 1979).

3.3 Characterization and identification of the isolates

3.3.1 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.

3.3.2 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 Biomerieux Vitek 2 System (Nucleus Diagnostic Centre, Kalyan).

3.4 Bioremediation

All Strains isolated during the course of study were investigated for their bioremediation activity. The screening was done by using 100ppm of heavy metals and by calculating MTC (Maximum Tolerance Capacity) for the isolate showing tolerance to 5 specific heavy metals i.e. Cd, Cr, Cu, Fe, Zn (5 heavy metals were chosen as these are common pollutant in industrial wastewater).

3.5 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/LATES
3.6 Mechanisms involved in Bioremediation

3.6.1 Isolation of Proteins

The bacterial cell of both the strains was grown at 45°C for 24 hrs. and the cells were harvested by centrifugation at 13,000 rpm for 10 minutes. 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 minutes. 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 heavy metals (control and Test). The most probable protein matching according to molecular weight was carried out by insilico method using by http://web.expasy.orgDatabase.

3.6.2 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.

The cell pellets from the culture were suspended in 10ml (20g /liter) NaCl in water and were centrifuged (6000 ×g, 30mins, 4˚C). Theresulting 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.

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-sulphuricacid method and were confirmed by FTIR analysis. The same method of isolation was performed with and without the treatment of heavy metals (control and Test) (Fabienne et al., 2011).

3.7 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 sodium chloride (salt). Salt is transparent to infrared light. The drop forms a thin film between the Plates.

4. Results and Discussion

4.1 Characterization of in situ Bioremediation

Microbial heavy metal reduction at high temperatures was studied at several sites in Vajreshwari & Ganeshpuri hot springs. Enrichment cultures were initiated with Bacillus Medium. After incubation of all enrichments for 6 days at in situ temperature, the cultures formed a dense colonies. Repeated transfer of enrichments revealed small rod-shaped 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 sterile media at 41°C. Stock cultures of both the strains were maintained on Sterile Media and transferred monthly fresh media.

4.2 Cellular properties

Cells appeared as very tiny straight rods. Motility was not observed. Exponential phase cells stained Gram-positive. Other biochemical properties (As per Berge’s manual) are described in Table 1, 2, 3. Species was again confirmed by using automated system of Biormerieux System (Done at Nucleus diagnostic Centre, Kalyan) stated as in Table 4 and 5.

Table 1: Biochemical Properties (According to Bergey’s manual)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Biochemical Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nitrate Cellulose Gelatin Casein H2S Catalase PAD MR VP Oxidase Citrate</td>
</tr>
<tr>
<td>SZP 1</td>
<td>+ - - - - - - + + - - +</td>
</tr>
<tr>
<td>SZP 2</td>
<td>- - - - - - - - - - -</td>
</tr>
</tbody>
</table>

4.3 Bioremediation

The MTC for both the strains for bioremediation of heavy metals were found to be above 1000 ppm, Table 6.

4.4 16s rRNA Analysis

The partial sequence analysis of conserved sites of both the strain gave the sequences. The sequences were aligned for comparison with the existing databases by using in silico tool BLAST. The isolate was found to be of genus *Thiobacillus* and type strain was found to be *Acidithiobacillus ferrooxidans* POPATNP (SZP 2), *Thiobacillus acidophilus* ABHAY (SZP 1).
Table 2: Acid Production and fermentation of Sugar (According to Bergey’s manual)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Biochemical Test ( Acid Production and fermentation of Sugar)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-Ara</td>
</tr>
<tr>
<td>SZP 1</td>
<td>+</td>
</tr>
<tr>
<td>SZP</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3: Physiological properties (According to Bergey’s manual)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Physiological properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>Temperature (oC)</td>
</tr>
<tr>
<td>SZP 1</td>
<td>7</td>
</tr>
<tr>
<td>SZP 2</td>
<td>5</td>
</tr>
</tbody>
</table>

Key: Growth: + ; No Growth: -

Table 4: Biochemical Tests (By Biomerieux Vitek 2 System) For SZP 1

<table>
<thead>
<tr>
<th>Biochemical Details for SZP 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 APPA - 3 ADO - 4 PyrA - 5 IARL - 7 d CEL - 9 BGAL -</td>
</tr>
<tr>
<td>10 H2S - 11 BNAG - 12 AGLTp - 13 d GLU + 14 GGT - 15 OFF +</td>
</tr>
<tr>
<td>17 BGLU - 18 d MAL + 19 d MAN + 20 d MNE + 21 BXYL + 22 BAlap -</td>
</tr>
<tr>
<td>23 ProA - 26 LIP - 27 PLE - 29 TyrA - 31 URE - 32 dSOR -</td>
</tr>
<tr>
<td>33 SAC - 34 d TAG - 35 d TRE - 36 CIT + 37 MNT - 39 5KG -</td>
</tr>
<tr>
<td>40 ILATk + 41 AGlu - 42 SUCT - 43 NAGA - 44 AGAL - 45 PHOS -</td>
</tr>
<tr>
<td>46 GlyA - 47 ODC - 48 LDC - 53 HISa - 56 CMT - 57 BGUR -</td>
</tr>
<tr>
<td>58 0129R - 59 GGAA - 61 IMLTa - 62 ELLM - 64 ILATa +</td>
</tr>
</tbody>
</table>

Table 5: Biochemical Tests (By Biomerieux Vitek 2 System) For SZP 2

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

Figure 1: Protein separation by SDS-PAGE SZP1
The strain sequence were deposited in NCBI database with KM527210 and KM527215 accession number respectively.

4.5 Protein Isolation
The gel showed that in presence of heavy metals some of the protein/enzyme for both the strains are over expressed. These proteins were identified by insilico method and most probable protein matching with the observed results are shown in figure 1 and 2.

The expasy results showed the probable proteins present may be, *Acidithiobacillus ferrooxidans* POPATNP (SZP 2):
1. Zn Transporter - 49.55 KD
2. Permease – 60.46 KD
3. Metal transporter – 49.51 kD
4. Iron transporter – 38.4 KD
5. Cadmium Transporter – 67.06 KD

*Thiobacillus acidophilus ABHAY (SZP 1).*
1. Zinc permease - 34 KD
2. Iron permease – 60.04 KD
3. Metal ABC transporter- 30.16 KD
4. Binding Protein – 26.48 KD

4.6 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 7 and 8) in case of test which was further confirmed by FTIR.

| Table 7: Amount of carbohydrate and protein present in EPS for Control and Test for strain SZP 1 |
| Carbohydrates concentration (mcg/ml) | Before and after exposure of 100 ppm of each heavy metal |
| Control | Cd | Cr | Cu | Fe | Zn |
| 28 | 31 | 28 | 29 | 28 | 39 |

| Protein concentration (mcg/ml) | Before and after exposure of 100 ppm of each heavy metal |
| 28 | 30 | 34 | 28 | 34 | 28 |

| Table 8: Amount of carbohydrate and protein present in EPS for Control and Test for strain SZP 2 |
| Carbohydrates concentration (mcg/ml) | Before and after exposure of 100 ppm of each heavy metal |
| Control | Cd | Cu | Cr | Fe | Zn |
| 30 | 34 | 35 | 30 | 35 | 30 |

| Protein concentration (mcg/ml) | Before and after exposure of 100 ppm of each heavy metal |
| 25 | 29 | 25 | 27 | 25 | 32 |

4.7 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 as shown in figure 3 and 4.

**Conclusion**

The present study extends the known niche for bioremediation in nature to extreme (> 60°C) thermal environments. Thermophilic organisms at high temperatures appears widespread in Vajreshwari&Ganeshpuri hot springs and one genus with two different strain and species was found to be associated with Bioremediation. Biochemical test (Done by Manual and automated method) and also 16s rRNA analysis showed that species found to be of *Acidithiobacillus ferrooxidans* POPATNP (SZP 2), *Thiobacillus acidophilus* ABHAY (SZP 1). Both the strains showed tolerance level of heavy metals above 1000 ppm.

The specified proteins were expressed during the bioremediation showed that various operons are responsible for this process. The chemical analysis of EPS and the FT-IR spectroscopy analysis confirmed the presence of proteins and carbohydrates. Further studies are required for identification and characterization of the same and for the use of these organisms for bioremediation of other heavy metals. As, the bioremediation of inorganic material is important for health of the people & for monitoring environment, in situ remediation by both *Thiobacillus species* is one of the best ways for the treatment of industrial effluent containing high levels of heavy metals.

**Research Highlights**

To the best of knowledge, this is the first report determining the mechanism responsible for heavy metal Bioremediation by *Thiobacillus* spp.
Isolation and identification of New strain of *Thiobacillus* Determination of presence of Extracellular Polymeric Substances Prediction of substances and protein responsible for heavy metal Bioremediation.

**Limitations**

Structure of EPS should be determined by hyphenated techniques.

**Recommendations**

Particular Protocol for isolation of EPS has to be used for different Species.

**Justification of Research**

The study will definitely help in improving knowledge of microbiology of hot water spring of Ganeshpuri and Vajreshwari, Maharashtra, India. The results will contribute for further structural and functional analysis of this protein and EPS.

**Author’s Contribution and Competing Interests**

The experimental work is accomplished by Sonali Zankar Patil under the guidance of mentor Dr. Geetha Unnikrishnan. Anju Unnithan helped in paper writing. We declare that we don’t have any financial or personal relationships that can create any conflicts of interests.

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**Very Special Finding**

Isolation and identification of New strain of *Thiobacillus spp.* Which were deposited in NCBI database.

**References**


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