Protein profiling of *heavy* metal tolerating new strain of *Pseudomonas* isolated from hot water spring

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ABSTRACT:

Thermophiles have developed a number of mechanisms to resist the toxic effects of heavy metals. The efficiency of bioremediation by thermophiles is dependent on the presence of biochemical factors present in the cell; like protein/ enzyme, EPS (Extra polymeric substances) or biogenic volatile substances. The first mechanisms by which bioremediation of toxic substances take place in microorganisms is with the help of protein/ enzymes. In the present study, heavy metal tolerating, new isolate of *Pseudomonas fluorescens SONALIZANKAR* was identified and the strain was deposited in NCBI database with accession number KM527212. The molecular mechanisms underlying the bioremediation phenomenon and the differential expression of proteins in response to the metal stress was also carried out. The band pattern in SDS-PAGE showed the difference in pattern of protein expression in presence and absence of heavy metals. Some proteins were over expressed in presence of heavy metals and some new bands were formed that indicates important role of proteins/enzymes during stress conditions.

Keywords: Thermophiles, protein profiling, Bioremediation, heavy Metals, NCBI.

I. INTRODUCTION:

Microorganisms, in the environment,break down organic matter to release mineral nutrients into a form that can be used by primary producers. For a large part of the geological past of the Earth, microorganisms particularly thermophiles play primary roles in shaping the natural environment by the enzymatic degradation of waste products to nutrient cycles, thus ensuring homeostasis of ecosystem. Microbial enzymes are proteins which can be studied in different natural ecosystems through the emerging field of meta-proteomics (The proteomic analysis of mixed microbial communities). One of the dissimilative reactions that thermophiles perform to gain energy and to decompose organic matter include aerobic respiration (oxygen reduction), nitrate reduction, sulfate reduction and metal reduction (Lynch and Hobbie 1988). However, when the environment become contaminated with a pollutant such as a heavy metal, some thermophiles will die while others are capable of survival. Thermophilic bacteria which survive are often capable of continuing these reductive reactions thereby degrading or altering the pollutant.

Thermophiles have proteins that are able to withstand high temperature. The search and production of these thermostable protein would generate new horizons for their application and generation of novel and value based products in the process of Bioremediation.

Proteomic analysis of these environmentally important thermophiles includes the direct qualitative and quantitative assessment of the full complement of proteins present in the microbes. Since thermophiles which are capable of bioremediation ,exist within a complex-heterogeneous environment, it is essential to develop a robust and effective protocol to extract proteins which are expressed by the thermophilic bacteria.

The protein profiling of heavy metal induced whole cell proteins of all the selected isolates was studied for finding out the metabolic changes if any, that can be reflected by molecular technique like Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). Differences seen in protein bands have been successfully used to study protein variation in response to various metal stress conditions (Krech*et al*., 1988; Huey and Hall, 1989).

II. RESEARCH METHODOLOGY :

The effect of heavy metals on the whole cell proteins of the selected isolates were studied using SDS- PAGE and proteins were identified by insilico method.

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.

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 *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 :

One 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).

4 *Bioremediation:*

Strain 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).

5 *Strain identification :*

It was carried out 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.

6 Estimation of proteins before and after exposure to heavy metals:

The selected isolates were grown in Sterile Nutrient Broth without heavy metals (Control) and with 100 ppm of heavy metals (Cd, Cr, Cu, Fe, Zn) (Test) separately. 10 ml of broth culture was removed from each flasks and centrifuged at 8,000 rpm at 4^{o}C± 2^{o}C for 10mins. The supernatant was discarded and the pellet was dissolved in 1ml of lysis buffer. The pellets were sonicated for 3 mins (with gap of 30 secs after each 1 min). The samples were centrifuged at 6000 rpm at 4^{o}C± 2^{o}C for 5mins. 250µl of the sample was mixed with 500 µl chilled acetone for 30min at 4^{o}C± 2^{o}C. the precipitated protein was dried. 100 µl PBS was added to dissolve the

precipitated protein (Isarankura-Na- ayudhya, *et al*., 2009). Folin Lowry's method was used for the protein estimation (Lowry, *et al.*, 1951) using std. BSA (250 µg/ml).

7 Isolation of Proteins :

All the selected isolates were grown in Sterile Nutrient Broth without heavy metals (Control) and with 100 ppm of heavy metals (Cd, Cr, Cu, Fe, Zn- monospecies and mixed heavy metal) (Test) and incubated was at 45ºC± 2ºC for 24 hrs. 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).

8 Protein separation and Protein identification:

The isolated proteins were subjected to SDS-Polyacylamide Gel Electrophoresis (SDS-PAGE) containing 2.5% stacking and 12.5% resolving gel casted on vertical gel electrophoresis apparatus (Technosearch) and separated using buffer under a constant voltage of 100V for 3 hrs. The higher molecular weight protein marker (BangloGenei) was simultaneously run to estimate the molecular weight of protein bands. After electrophoresis the gel was kept for overnight stained with Coomassie Brilliant Blue R-250 (Hi Media) prepared in methanol and glacial acetic acid. The gel was destained with methanol and acetic acid mixture. The stained bands were visulised and compared with protein marker (Sawhney and Singh, 2009).

These proteins were identified by insilico method and most probable protein matching with the observed results were analysed by insilico method using http://web.expasy.orgDatabase.

III. RESULT AND DISCUSSION :

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 d 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 mucoid colony (SZP 12) when cultured on st. media at 41°C. Stock cultures were maintained on St. Media and transferred monthly.

Cellular properties :

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

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

Isolate		Biochemical Tests										
	Nitrate	Cellulose	Gelatin	Casein	H_2S	Catalase	PAD	MR	VP	Oxidase	Citrate	I
SZP 12	+	-	+	-	-	+	+	-	-	+	-	+

Table 2: Acid Production and fermentation of Sugar (According to Bergey's manual)

Isolate	Biochemical Test (Acid Production and fermentation of Sugar)										
	L-Ara	Glu	Gal	Suc	Mal	Fru	Raf	Star	Cel	Ino	Mann
SZP 12	+	+	-	+	+	+	+	+	-	+	+

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

Isolate	Physiological properties		
	pH	Temperature (ºC)	NaCl (%)
SZP 12	7	45	2

Key : Growth : + ; No Growth : -

Table 4 : Biochemical Tests (By BiomerieuxVitek 2 System) For SZP 12

Biochemical Details for SZP 12																	
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	MAL	-	19	dMAN	-	20	MNE	-	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	-			

Bioremediation :

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

Table 6: MTC for heavy Metals

Isolate	Maximum Tolerance Capacity for Heavy metal (concentration in ppm)				
	Cd	Cr	Cu	Fe	Zn
SZP 12	4389	2356	4430	2977	3972

16s rRNAAnalysis :

The partial sequences was aligned for comparison with the existing databases by using in silico tool BLAST. The isolate was found to be ***Pseudomonas fluorescens with*** new strain, the sequence of which was deposited in NCBI database with **KM527212** accession number and strain name given was ***SONALIZANKAR*** *(SZP 12).*

Estimation of proteins before and after exposure to heavy metals:

The isolate exposed to 100ppm of five selected heavy metals showed an increase in cellular proteins compared to the control (Table 1, Figure 1). The results indicate that the proteins/ enzymes required for the uptake of heavy metal salts within the cell increases in the presence of heavy metals. There was an approximate 1 to 14 µg /ml increase in the concentration of bacterial cell protein sin response to heavy metal uptake.

Table 7 : Estimation of proteins before and after exposure to 100 ppm of Heavy Metal Solution

	Protein concentration (µg /ml) Before and after exposure of 100 ppm Cd^{2+}				
	Cd	Cr	Cu	Fe	Zn
Control	28±2.5	28±1.0	28±1.3	28±1.8	31±1.7
Test	29±2.8	36±1.6	40±1.1	38±1.9	35±1.5

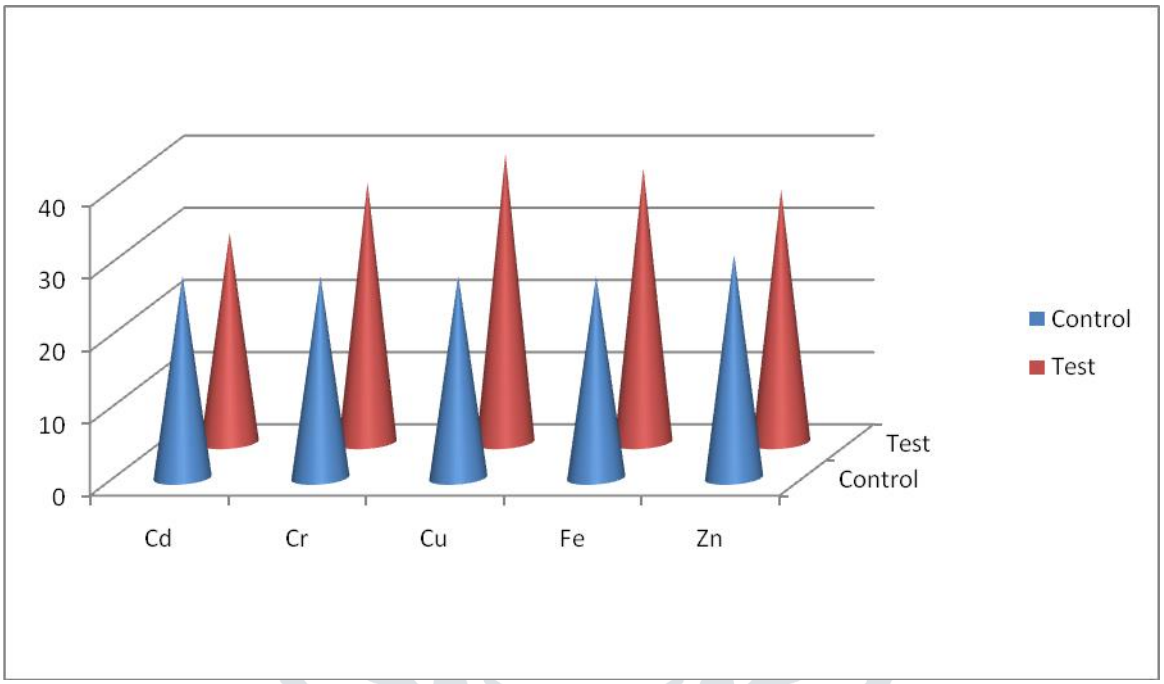

Figure 1: Estimation of proteins before and after exposure to 100 ppm of Metal Solution

Protein separation and Protein identification:

Protein profiling of the isolate in the presence of 100 ppm of heavy metals (monospecies and mixed heavy metal) showed over expression of some of the proteins/ enzymes as compared to control (Figure 2). The molecular weights of each protein expressed were calculated using marker.

The presence of various bacterial protein bands which were expressed when the cells were grown in the heavy metal salts were observed. There was either thickening of the protein band which was also present in the control or there was a new band formation when the isolates were exposed to heavy metals. This indicates that the protein concentration has increased in the cell in the presence of heavy metals.

The isolateSZP 12 haveover expressed proteins like transporter (Ranging from 9.02 KD to 65.93 KD). It has showed presence of some regulatory protein and repressor protein in addition to transporter (Table 2).

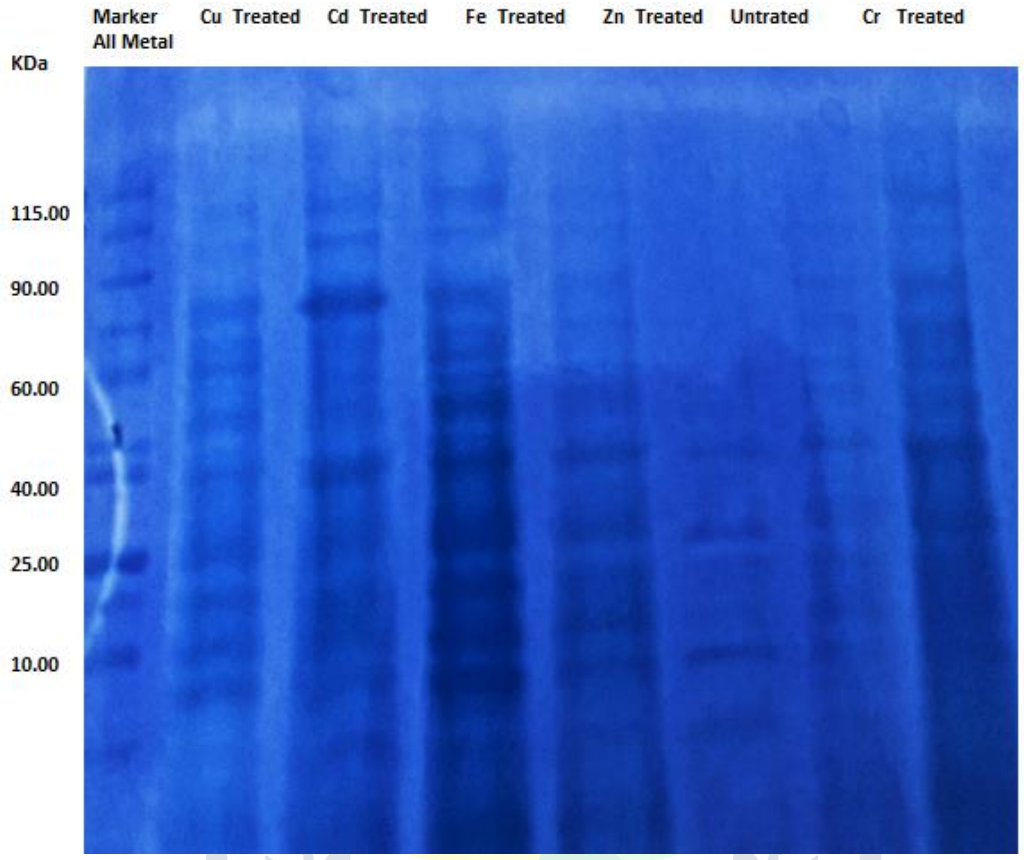

Figure 2: Protein separation from *Pseudomonas fluorescens SONALIZANKAR (SZP 12)*

Table 8: The expasy results showed the probable proteins present may be
Pseudomonas fluorescens SONALIZANKAR (SZP 12).
1. Cadmium transporter ATPase - 65.93 KD
2. Copper Transporter - 42.07 KD
3. Ferrous iron transporter - 31.88 KD
4. Periplasmic iron transport protein - 42.96 KD
5. Zinc transporter - 28.21 KD
6. Cobalt- Zinc- Cadmium resistant protein - 9.04 KD

IV. CONCLUSION:

Thermophiles adapt to change their lives with unique "survival strategies" available with them. It has been realized that thermophiles have developed diverse metal resistance mechanisms to adapt with high concentration of dissolved metals associated with their extreme environmental living conditions. **T**he study of molecular mechanisms underlying the bioremediation phenomenon and the differential expression of proteins in response tothe metal stress was carried out. The introduction and over expression of metal binding proteins increases the metal binding capacity, tolerance or accumulation of heavy metals into the cells of the isolates.

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