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Bioremediation of Heavy Metal using Growing Cells in Industrial Effluent

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ABSTRACT: Removal of heavy metals from contaminated domestic-industrial effluent using eight resistant indigenous bacteria isolated from acclimatized activated sludge was investigated. Proper standard protocols were followed to study the bioremediation of heavy metals using growing cells in industrial effluent. They were identified as *Bacillus pumilus, Bacillus subtilis, Pseudomonas aeruginosa, Serratia liquefaciens.* It is therefore recommended that the proposed process augmented with the acclimatized strains is the best choice to ensure high treatment efficiency and performance under metal stresses especially when industrial effluents are involved.

Keywords: Heavy metals, industrial effluent, bioremediation, microbes

I. INTRODUCTION

It can be assumed that the selective pressure exerted by heavy metals contribute to the indirect co-selection of antibiotic resistance [1] particularly in environments contaminated with the two element [2]. Concerning heavy metals, terms such as "resistance" and "tolerance" are arbitrary and they are often used as synonymous in literature [3]. Gadd [4] suggested using the term "resistance" when it is possible to characterize a specific mechanism of bacterial detoxification for a metal. Therefore, the term tolerance seems more appropriate to refer to the ability of a bacterial strain to grow in the presence of high concentrations of a metal, in all cases in which the mechanism of this process has not been investigated [5]. The toxic effects of heavy metals on microorganisms are influenced by a multitude of factors such as pH, concentration of chelating agents, speciation, and organic matter [6].Uncontrolled discharges of large quantity of heavy metals containing waste create huge economic and health care burden, particularly for the people living near that area [4]. The toxic metal pollutant like Lead, Nickel and Cadium enter to the water bodies through industrial waste water [7]. Among the heavy metal, Lead is a nonessential heavy metal and general toxicant. The toxicity of these heavy metal occur through the displacement of essential metal from their native binding site or through ligand interaction [8, 9]. The toxicity can occur as a alteration in the confirmational structure of the nucleic acid and protein and interference with oxidative phosphorylation and osmotic balance [10]. The microbial flora responds to these heavy metals by several processes [11] including the transport across the cell membranes [12] bio-sorption to the cell walls [13] and entrapment in extracellular capsules (Sharma 2014) precipitation, complexation and redox reactions [14, 15]. Microbial collection of bacteria, fungi and algae both in live and inactivated form are reported to be capable of removing hazardous heavy metal ions [16] by two well known process mechanisms i.e., (i) biosorption: binding of metal ions to cell walls devoid of energy dependency and (ii) bioaccumulation: an energy- dependent process of metal uptake into the cells [17, 18]. They can be single metal resistant or multi- heavy metal resistant depending on their genomic sequence variability. A single bacterial strain can be found to be resistant to many metals [19, 5]. A bacterium isolated on the basis of tributyltin resistance was found to be resistant Heavy metals are not biodegradable and tend to be accumulated in organism and because of numerous disease and disorder [20]. To survive under metal - stressed condition, bacteria have evolved several types of mechanism to tolerate the uptake of heavy metal ions [21].

These metal mechanism include the efflux of ions outside the cell, accumulation and complexation of metal ions inside the cell [22]. Heavy metal can damage the cell membrane, alter enzyme specificity, distrupt cellular function and damage the structure of the DNA [23]. The toxic effect of Arsenic, Mercury and Lead were known to be ancient, but methodical studies of the toxicity of some heavy metal appear to date from only 1868 [24] in human ,heavy metal poisoning is generally treated by the administration of chelating agents [25]. Additionally, metal tolerant Enterobacteriaceae strains were investigated for their resistance to antimicrobial drugs [17] intending to study the possible relationship between metal tolerance and antimicrobial resistance. The toxic effects of heavy metals on microorganisms are influenced by a multitude of factors such as pH, concentration of chelating agents, speciation, and organic matter. There are significant practical limitations to biouptake by living cell system such as sensitivity of the system to extremes of pH, high metal/salt, concentration and requirement of external metabolic energy. the isolation and selection of metal resistant aspect to overcome the prime constraint of employing living cells systems, incidentally resistant cells are expected to bind substaintially more metals which in turns is a prerequestie for enhanced bioprecipitation/intracellular accumulation and development of efficient process [26]. There was a rapid accumulation of Cd in the first 2 days of the 6-th day growth period. At highest concentration (45 mg/l),the accumulation continued and a maximum was recorded on the fifth day of the culture. Thus, the response of cells to metal stress depends on the ambient concentration of metal [27]. This study aims to obtain data about bioremediation of heavy metal using bacteria and its estimation in the effluent before and after treatment. The study further investigated impact resulting from the interactions between metals and metal tolerant bacteria.

II. MATERIALS AND METHODS

A. Sampling

Five heavy metal contaminated effluent samples were collected from different stations at five sampling sites and were immediately transferred to the laboratory in the pre-sterilized bottle containers. All the samples were kept at refrigeration (4°C) till processing. Applicable sample was processed immediately for microbiological studies.

1. Estimation of heavy metals. The methodology of [28] was used for the estimation of heavy metals. The prepared samples were allowed to stand overnight to slow mineralization. Then the samples were mineralized in a hot plate. Heavy metal concentration in the dried samples were estimated using AAS 7000 spectrometer (Shimadzu, Japan) with graphite furnace atomization (GF-AAS) for Chromium (Cr), Nickel (Ni), Lead (Pb), Copper (Cu) and Manganese (Mn) or flame atomization (F-AAS) for Zinc (Zn).

2. Isolation of distinct morphological bacteria. Selected heavy metal contaminated effluent sample was serially diluted and spread plated on distilled water prepared nutrient agar plates. After 48 hrs incubation at 37°C, the distinct morphological bacterial strains were pointed and pure cultured repeatedly using quadrant strike plate method in fresh distilled water prepared nutrient agar plates. The purity of the strains were concluded using distinct colony morphology and gram staining procedure.

3. Screening of promising heavy metal resistant bacteria. All the axenic strains were individually cultured in 250ml conical flask containing 100ml pr-sterilized heavy metal contaminated effluent (sample 1) sample supplemented with 1% glucose and 0.5% peptone as carbon and nitrogen source along with the basal cultural conditions of 37°C temperature with 150 rpm agitation. Strains showed maximum growth on the basis of dry weight of cell biomass (g/L) against the heavy metal contaminated effluent was chosen for further heavy metal bioremediation studies. For the dry weight estimation of bacterial cells, the axenic cultured broth after 48 hrs was centrifuged at 3000 rpm for 15min which were kept in hot air over at 50°C for an hour.

Bacterial consortium was developed using the promising heavy metal resistant strains which were further used for heavy metal tolerance and bioremediation studies.

4. Growth standardization of bacterial consortium. The promising heavy metal resistant bacteria consortium was standardized for growth conditions by adopting search technique, *i.e.,* varying one parameter at a time. The value of a particular parameter achieved by one step was fixed in subsequent experiments. The fermentation process was carried out in a 2L conical flask with 800ml working volume using the broth as the basal cultural conditions of 1% glucose, 0.5% peptone, pH 8.0, 34°C temperature and agitation at 150 rpm.

The inoculum was prepared using the exponential phase culture of this promising consortium in the same cultural conditions, where the optical density (OD 620 nm) of the inoculum culture was adjusted to 0.1 based on McFarland turbidity 0.5 standards which were equivalent to the bacterial concentration of 1×10^8 cfu/mL. Factors like heavy metal tolerance, carbon source, nitrogen source, pH, temperature and agitation were tested in different ranges with the growth of bacterial consortium, estimated using dry weight of cell biomass (g/L) as determined earlier in screening studies of promising heavy metal resistant strains.

5. Heavy metal tolerance. Four heavy metals which were at higher concentrations in the collected effluent, they were *viz* chromium, nickel, lead and copper whereas zinc and manganese were considerable low in their presence. So, the heavy metal tolerance of the bacterial consortium was studied with these four heavy metals which were used at the range between 25ppm to 150ppm with an interval of 25ppm. Using each of four heavy metals, the tolerance was examined at 100ppm to 600ppm with intervals of 100ppm. The used heavy metal substitutes were chromium chloride, nickel sulfate, lead (II) phosphate and copper sulfate. Heavy metal substitutes were prepared at the concentration using the formula (X);

X = Molecular weight of compound/ Molecular weight of heavy metal × amount of sample required (ppm)

6. Heavy metal bioremediation using bacterial consortium. The heavy metal contaminated industrial effluent was treated with the bacterial consortium developed in this study with the standardized growth conditions except the heavy metal tolerance parameter. The bacterial consortium was identified for its peak time of heavy metal bioremediation with reference to its cell growth. The enhanced bioremediation was monitored using a portion of cultured broth followed by the separation of cell free supernatant and cell pellet using centrifugation at 3000rpm for 15 min. The fermentation process was monitored for 102 hrs with an interval of 6 hr starting from the lag phase to decline phase under batch culture conditions. Individually estimation was monitored for the bacterial growth using the dry weight of cell biomass (g/L) as described earlier and heavy metals in the cell free supernatant and cell pellet was determined using AAS as described below.

7. Estimation of heavy metals using AAS in the treated broth. Heavy metals in the cell free supernatant was determined with a 100ml added with 3ml HNO3 followed by complete dryness on a hot plate whereas the cell pellet was lyophilized or freeze dried. Further, the same applied conditions in the AAS for the estimation of heavy metals in the effluents were applied here for the determination of six heavy metals viz. Zn, Cr, Ni, Pb, Cu and Mn.

III. RESULTS

A. Study of heavy metal in effluent samples

All the samples were individually tested for their heavy metals contaminants using atomic absorption spectrometry (AAS). The amount of heavy metal are given in table 1. The effluent sample collected from Sample 1 showed highest heavy metal contaminations using AAS which was further proceeded for bioremediation studies.

Table 1: Qualitative and quantitative estimations of heavy metals in the collected different effluents sample.

S. No.	Heavy metals	Quantitative estimations (ppm) in different effluent samples				
		SAMPLE 1	SAMPLE 2	SAMPLE3	SAMPLE 4	SAMPLE 5
1	Zn	0.2	11.8	18	17	0.3
2	Ni	21	0.3	25	0.4	0.2
3	Mn	0.2	28	0.3	21	12
4	Pb	19.5	0.2	0.2	29	0.2
5	Cu	41	0.3	32	0.2	17
6	Cr	39	29.9	0.1	0.1	18

B. Isolation of appreciable heavy metal resistant bacteria

The purity of the strains were concluded using distinct colony morphology and gram staining procedure. It was identified as *Bacillus pumilus, Bacillus subtilis, Pseudomonas aeruginosa, Serratia liquefaciens.*

Table 2: Growth of bacterial consortium in different concentrations of heavy metals.

Dry weight of cell biomass (g/L)	5.67	5.52	5.45	5.39	3.57	1.89
Total heavy metal concentration (ppm)	100	200	300	400	500	600

The consortium showed consistent and appreciable heavy metals tolerance in the medium up to 400 ppm. growth of bacterial consortium using different carbohydrate sources at the appreciable heavy metals tolerance condition. The consortium showed enhanced growth of 7.59g/L in the medium replaced with yeast extract as the nitrogen source (Table 3).

Table 3: Growth of bacterial consortium using different nitrogen sources at the appreciable heavy metals tolerance condition.

Nitrogen		**Yeast**	**Malt**	**Beef**	**Ammonium**	**Sodium**
Source (1%)	Peptone	extract	extract	extract	sulphate	nitrate
Dry weight of cell biomass(g/L)	6.77	7.59	7.01	6.98	6.03	5.98

Table 4: Growth of bacterial consortium using various pH parameters at the appreciable heavy metals tolerance condition.

Various pH	6	6.5	7	7.5	8	8.5	9
Dry weight of Cell biomass(g/L)	6.34	7.05	7.71	7.83	7.6	5.67	2.45

The consortium showed enhanced growth of 7.83g/L in the medium replaced with pH 7.5 (Table 4).

Table 5: Growth of bacterial consortium under various temperature parameters at the appreciable heavy metals tolerance condition.

Different Temperature (°C)	25	30	35	40	45
Dry weight of Cell biomass (g/L)	5.72	7.45	8.05	7.31	5.69

The consortium showed maximum growth of 8.05g/L using 35°C (Table 5).

Table 6: Growth of bacterial consortium under different agitation at the appreciable heavy metals tolerance condition.

Different Agitation (rpm)	0	50	100	150	200	250
Dry weight of cell biomass (g/L)	6.33	7.21	7.7	8.06	7.8	7.05

The consortium showed maximum growth of 8.06g/L using agitation of 150rpm. Individually estimation was monitored for the bacterial growth using the dry weight of cell biomass (g/L) as described earlier and heavy metals in the cell free supernatant and cell pellet was determined using AAS as described in (Table 6). There was low amount of heavy metal bioremediation observed till the end of log phase as shown in table 7, but revealed appreciable amount of heavy metals bioremediation observed at the end of stationary growth phase of consortium which was reflected from the least amount of heavy metal presence in the effluent broth at the end of stationary phase. Moreover, at the decline phase cell started to die and reintroduce the heavy metals to the broth.

Table 7: Quantitative analysis (ppm) of heavy metals in cell free supernatant of treated effluent at different growth phases of bacterial consortium.

	Quantitative analysis (ppm) in cell free supernatant of treated broth		
Heavy metals	**End of log phase culture**	**End of stationary phase culture**	**Middle of Decline phase culture**
Zn	0.1	0.05	0.1
Ni	17.5	0.97	12
Mn	0.1	0.03	0.1
Pb	16	0.57	7.7
Cu	31.3	3.03	22
Cr	32	3	14.5

The presence of highest quantity of heavy metals in the cell pellet analyzed at the end of stationary phase showed the maximum heavy metal bioremediation at that growth phase which were not observed in log and decline phases as shown in table 8. All together, at the end of stationary growth phase the bacterial consortium revealed maximum heavy metal bioremediation. Maximum (93.67%) heavy metal bioremediation was predicted at the end of stationary growth phase of bacterial consortium (Table 9).

Table 8: Quantitative analysis (ppm) of heavy metals in cell pellet of treated effluent at different growth phases of bacterial consortium.

Heavy metals	Quantitative analysis (ppm) in cell pellet of treated broth		
	End of log phase culture	End of stationary phase culture	Middle of Decline phase culture
Zn	0.1	0.14	0.1
Ni	3.3	20	8.8
Mn	0.1	0.16	0.1
Pb	3.3	18.9	11.6
Cu	9.4	37.94	18.7
Cr	6.7	36	24.2

Table 9: Percentage estimation of heavy metal bioremediated at different growth phase of bacterial consortium.

Growth phases of bacterial consortium	Log phase	Stationary phase	Decline phase
Percentage heavy metal bioremediated	19.77%	93.67%	53.35%

IV. DISCUSSION

Metal biosorption by microbial biomass is a notable progressed biotechnological instrument utilized for effective evacuation of contaminant metals. Improvement instruments of metal biosorption included numerous variables among which pH is the most essential one [29] and additionally the C/N proportion of initiated muck [30] and temperature [31]. Acclimatization is a critical procedure for procuring new propelled highlights of microorganisms that can be proficiently connected in metal biosorption. Amid the present investigation, utilizing strains already accustomed by presenting microbial biomass to metal focus angles [28] demonstrated high proficiency in the improvement of actuated slop execution for metals and natural issue evacuation even at high metal burdens. For instance, *Enterobacter* sp. (Cu1) adjusted to get by under elevated amounts of Cu stretch (200 mg/l) indicated high effectiveness for Cu (31.37 Cu/g biomass) and natural issue (58.10 %) evacuation contrasted with 2.93 mg Cu/g accomplished as the most extreme expulsion by plain actuated muck [32]. Comparable outcomes were acquired by controlling metal restricting limit of *Nocardia amarae* cells to upgrade the general Ni, Cu and Cd restricting limit of actuated muck [33]. As in the present investigation the unadulterated culture of *Nocardia* displayed altogether higher metal sorption limit than the enacted slime biomass credited to the way that the *Nocardia* cells developing at stationary stage have generously more particular surface territory than that of actuated muck. The metal sorption limit of actuated muck expanded relatively with the measure of *Nocardia* cells introduce in the blended alcohol [34]. Sirianuntapiboon and Ungkaprasatcha likewise revealed proficient expulsion of Pb and Ni and in addition natural issue by contrasting acclimatized and un-acclimatized biosuldge.

V. CONCLUSION

Qualitative and quantitative estimation of analysis of heavy metal present in the collected different industrial effluent sample was found to be higher before the treatment, one of the heavy metal contaminated sample I was selected for further treatment. The characterization of metal resistant bacteria was done .Growth standardization of consortium in the medium using the highest heavy metal tolerance condition was done. It was reduced after treatment with bacterial consortium and it was proved by quantitative analysis of heavy metals in cell free supernatant of treated effluent at different growth phases of bacterial consortium and quantitative analysis of heavy metals in cell pellet of treated effluent at different growth phases of bacterial consortium.

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