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Chromium (Cr) Biosorption, from High Energy Battery (Heb) Effluent Using Fungi

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Abstract

In the current industrialized world, uses of batteries have gained importance as a result of its high performance and energy storage system. Though, battery was considered safe and produced less carbon foot print, it also has its ill effects such as heavy metal contamination in water and soil, which is also at its threshold to be addressed. Here comes the concern, about the elevated heavy metal concentration especially chromium in the environment and its health hazards exerted over all living organisms. Thus, as an approach towards the bio sorption of chromium from environment, fungal isolates obtained from soil samples of HEB effluent were screened for their resistance and efficiency. Among the 36 fungal isolates attained, *Aspergillus* was found to be most predominant in both eastern and western area, as well as more resistant to chromium even at 1000 ppm. On further optimization of pH, Temperature, incubation period, carbon source, nitrogen source and phosphorous, it concluded that medium with Glucose, malt extract and potassium dihydrogen phosphate at pH 5 showed significant growth and proficient absorption of chromium under static condition. The 18s rRNA gene sequencing of the effectual organism revealed to be *Aspergillus niger* (KY354579) with 99 % according to BLAST analysis.

Keywords: Industrialization; Environmental Contamination; Health Hazards; Resistance; Growth Factors; *Aspergillus spp.*

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Introduction

Batteries have become the most important component in day-to- day activities of human life, especially in the fields of transportation and grid applications due to its high energy storage system [1]. Customization of batteries was particularly emphasized as a realistic solution towards the sustainable energy storage system, in order to combat the issues faced by fossil fuel consumption and its effects on environmental deterioration [2]. Rather, batteries also possess harsh effects to the environment by their heavy metal effluent leakage, which need to be focussed immediately in order to save the future environment, as well as generations. Major flaw lies in the release of heavy metals into the soil and water, which leads to health concerns among all living organisms throughout water and soil pollution [3].

Among many heavy metals, Chromium lies amidst the most interested, as it possesses both boon and flaw (i.e.) it is highly

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recommended in industries as a tool of economic growth, as well as it's tremendously toxic to the environment [4]. Chromium, even at minute concentrations in high energy battery (HEB) effluent are considered to be most dangerous as it is highly carcinogenic and mutagenic due to their increased solubility, cellular protein interaction and biological membrane permeability [5]. Moreover, Chromium (VI), the hexavalent state is extremely toxic due to its ability to pass through the cell membranes and their transportation to the interior of the cell through sulfate transporter, by which they attain more solubility and mobility in the soil and aquatic ecosystems. Among other heavy metals chromium is ranked within the top sixteen [6], considered to be the source of high-risk disease such as nephrotoxic malignant neoplastic disease [7]. Besides this, Cr (VI) creates abiotic stress up on plants and animals heading towards environmental imbalance [8]. Furthermore, Thyagarajan stated that electroplating industry disposes around 100 mg/l of Chromium, which is highly hazardous [9]. Moreover, heavy metals particularly, Chromium are expelled into this environment through various other means such as leather tanning, pigments, dyes production, metal finishing, electroplating, metallurgy, battery manufacturing, and wood preservation [4]. All these effects are deteriorating the current ecosystem, which needs to be managed instantly, as an approach of lending hand to the future generations, by means of management technologies.

Waste water and effluent treatment by conventional methods, including solvent extraction, adsorption using polymeric resins, biopolymers, activated carbon and graphene oxide are available but still, they have not gained importance due to their extensive cost, energy consumption, decreased efficiency, production or raised secondary waste which is awfully difficult to dispose [10]. On overcoming these hindrances, novel methods such as bio sorption of metals using microbes need to be invested which could reduce metal contamination at a low cost and increased efficiency. Proficiency in metal absorption by microbes can be easily obtained, as the functional groups and active binding sites in cell surface of microbial cell wall capably uptakes heavy metals [11]. Many organisms including bacteria, fungi and algae isolated from chromium polluted soil and water have also proven to be effective based on their optimal growth conditions and also concentration of contamination. Moreover, Bhattacharjee et al has stated that the effect of microbes on effluent bio sorption vary drastically according to the environmental conditions of their regions. Therefore, optimization of environmental parameters such as pH, temperature, carbon source, nitrogen source etc., is essential to determine the effect of most degradative organisms in the polluted soils. Consequently, this study is concentrated on fungal isolation from polluted samples and further optimization of environmental parameters to obtain efficient degradative microbes [12].

Materials and Methods

Study site's description

Avoor, a village in Viralimalai taluk of Pudukkottai district, Tamilnadu, India, was selected as two sampling sites, where increased concentration of heavy metals and their by-products are released into the environment. It is located 45 Km West to the District headquarters of Pudukkottai, one among the least semi-urbanised districts of Tamilnadu. Its latitudinal and longitudinal geographical positions are 10.163603^{0}N and 78.332391^{0}E, respectively.

Sample collection

Soil samples at a depth of 15 cm from the surface of study site, were collected in random locations at a particular time, in triplicates. Collected samples were packed in sterile polythene bags, air dried and stored at 4°C for future processing. Quantitative analysis for heavy metal contamination, in the sample was conducted at Trichy soil testing laboratory, Tamilnadu, India.

Fungal isolation from sample

Ten grams of the soil sample were mixed in 100ml of sterile distilled water and were serially diluted up to 10^{-5} dilutions. One ml of 10^{-3} dilution was spread over Potato dextrose agar plate (pH 5.6) containing streptomycin sulphate (100 mg/L^{-2}), to prevent bacterial growth and then the plates were incubated at 25±2°C for five days. Fungi was further identified morphologically, based on their colony morphology and reproductive structural characteristics like sporangiospore position, columella and spore shape followed by lactophenol cotton blue staining technique [13].

Heavy metal tolerance assay

Fungal isolates, in order to determine their heavy metal tolerance was spot inoculated in freshly prepared potato dextrose agar medium containing different concentrations of potassium dichromate, ranging from 100 to 1000 ppm. On further incubation of 24 hours, the mycelial growth of fungal isolates were monitored for the effect of chromium on it. Metal tolerance index (Ti) of those isolates, were determined using the formula; Ti= Dt/Du x100 where, Dt is the radial extension of treated colony in cm and Du is the radial extension of untreated colony in cm.

Fungal bio sorption

1000 mg of potassium dichromate was dissolved in 1L of deionized water to obtain the chromium stock solution. Two sets of chromium ion solution were prepared from stock at a concentration of 50 mg/L (pH 7). Each 100 ml of chromium ion solution was inoculated with 0.5 g of live and alkali-treated fungal biomass and the reaction mixture were incubated in shaker for 60 minutes, after which the chromium concentration was estimated.

Estimation of chromium

5 ml of the reaction mixture was centrifuged at 4000 rpm for 15 min, to obtain the supernatant. To 1 mL of the supernatant, 9 mL of 0.2 M sulphuric acid and 0.2 mL of 0.25% diphenyl carbazide in acetone were added, thereby which the solution turns pink. Using distilled water as blank, the Optical density of the solution was identified at an absorbance of 540 nm. Furthermore, the amount of chromium was estimated using linear regression of the standard graph and its percentage was calculated using the formula; E = (Ci - Cf / Ci) × 100 where, E = Percentage removal of heavy metal; Ci = initial metal ion concentration, mg/L and Cf = final metal ion concentration, mg/L.

Effect of nutrient source on bio sorption

Bio sorption efficiency of chromium, which varies significantly with the effect of nutrient sources such as carbon and nitrogen were analysed. Chromium containing medium, with different carbon sources including glucose, sucrose and mannitol at a concentration of 1 g/L were prepared for identifying their efficiencies. Similarly, the potato dextrose broth containing 0.01% phosphorus of ammonium ortho phosphate, potassium dihydrogen phosphate and potassium dihydrogen monophosphate were prepared, to which the nitrogen sources which includes sodium nitrate, malt extract and peptone were altered at 0.1% and incubated for 7 days at a constant pH 6. Post incubation, the percentage of heavy metal accumulation was estimated in comparison with control and the biosorption efficiency (%) was calculated by the formula, described above. Moreover, microbial growth was also observed with varying pH of 5, 6.8 and 9 with Malt and glucose as they showed much greater efficiency at constant pH.

Molecular analysis of fungi

Fungal genomic DNA extraction using spinwin column method was carried out using 50 to 100 mg of homogenized fungal mycelia/spores. Further the molecular characterization was attained, based on the 18S rRNA gene and internal transcribed spacer regions (ITS1-5.8S-ITS2) [14] amplified using universal fungal primers such as ITS1 5-TCCGTAGGTGAAC CTGCGG-3; ITS4 5-TCCTCCGCTTATTGATATGC-3; forward universal 18S rDNA primer NS1 (5'- GTAGTCATATGCTTGTCTC-3'); reverse 18S rDNA primer C18L (5'- GAAACCTTGTTACGACTT-3'). Purification of PCR products were carried using purification kit and then subjected to sequencing to analyse the strain using BLAST with NCBI database. Secondary structure of the selected fungal strain was predicted using Gene bee structure prediction software (/service/ma2-reduced.html). Accordingly, the restriction sites in 18S rRNA and ITS regions of selected fungal stain was analysed using NEB cutter program version 2.0 tools online (.com.NEBCutter2/index.php).

Table 1. Concentration of Heavy metal contamination from collected samples.

Heavy metal	Concentration in Eastern areas (mg/Kg)	Concentration in Western areas (mg/Kg)
Chromium	127±1.3	124±1.5
Cadmium	0.1±0.03	0.5±0.28
Nickel	0.9±0.2	1.1±0.2
Lead	5.4±0.96	7.9±0.24
Zinc sulphate	7.9±1.6	6.2±0.15
Copper sulphate	8.5±1.9	8.1±0.02
Magnesium sulphate	7.1±1.8	8.5±2.03

Discussion

Soil samples collected from western and eastern areas, of battery effluent contaminated fields in Avoor, Pudukottai showed the presence of many heavy metals. Additionally, the eastern areas were detected with increased Chromium contamination in comparison with western areas which is depicted in (**Table 1**). Ayele and Godeto [11] has also reported the presence of Chromium at higher concentration along with other heavy metals such as Cobalt, cupper, cadmium, arsenic, gallium, germanium, iron, mercury, lead, nickel, thallium, selenium and manganese. Moreover, states like Tamil Nadu, Maharashtra and West Bengal indicate an alarming rate of Chromium present in soil and ground water [15].

Microbial analysis of sample showed the existence of 112 isolates from eastern area and 84 isolates in western area. Further findings showed that *A. versicolor* and *Cladosporium* sp. were

Table 2. Isolated fungal diversity and simpson index.

Name of the Fungi	ni	N	pi	Logpi	Shannon	ni/N	Simpson	Abundance	Density
Alternaria alternaria	3	196	0.015306	-1.81513	-0.02778	0.015306	0.000234	196	98
Aspergillus flavus	20	196	0.102041	-0.99123	-0.10115	0.102041	0.010412	196	98
A. fumigatus	23	196	0.117347	-0.93053	-0.10919	0.117347	0.01377	196	98
A. niger	30	196	0.153061	-0.81513	-0.12477	0.153061	0.023428	196	98
A. versicolor	2	196	0.010204	-1.99123	-0.02032	0.010204	0.000104	196	98
Cladosporium sp.	3	196	0.015306	-1.81513	-0.02778	0.015306	0.000234	196	98
Curvularia lunata	5	196	0.02551	-1.59329	-0.04065	0.02551	0.000651	196	98
Fusarium semitectum	26	196	0.132653	-0.87728	-0.11637	0.132653	0.017597	196	98
F. oxysporum	5	196	0.02551	-1.59329	-0.04065	0.02551	0.000651	196	98
F. solani	7	196	0.035714	-1.44716	-0.05168	0.035714	0.001276	196	98
Penicillium citrinum	29	196	0.147959	-0.82986	-0.12279	0.147959	0.021892	196	98
Rhizopus sp.	1	196	0.005102	-2.29226	-0.0117	0.005102	2.6E-05	196	98
R. oryzae	3	196	0.015306	-1.81513	-0.02778	0.015306	0.000234	196	98
Trichoderma harzarnum	30	196	0.153061	-0.81513	-0.12477	0.153061	0.023428	196	98
T. viride	6	196	0.030612	-1.5141	-0.04635	0.030612	0.000937	196	98
Verticillium sp.	3	196	0.015306	-1.81513	-0.02778	0.015306	0.000234	196	98
Total Number of species	196		Index	Shannon	-1.0215	Simpson	0.11511	3136	1568
						Pielous Evenness index		e=H/logS	-0.083018

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completely absent in the eastern area where similarly, *Alternaria alternata, Curvularia lunata, F. oxysporum, Rhizopus* sp. *T. viride* and *Verticillium* sp. were completely absent in western area. The Shannon index, Simpson index and Pielou's Evenness index for the understanding of fungal diversity were identified to be -1.0215, 0.11511 and 0.083018 respectively, which is tabulated in (**Table 2**). Accordingly, [16] has also confirmed the presence of *Aspergillus flavus*, *Aspergillus niger, Fusarium solani,* and *Penicillium chrysogenum* in peri-urban agricultural areas, their adaptations and removal of heavy metals in industrial effluent, municipal sewers and mining sites. *Aspergillus niger* and *Penicillium chrysogenum* isolated from municipal sewage were found to tolerate lead, copper and cadmium at a concentration of 800 ppm [17].

Current study proves the resistance of five fungal species (i.e.) *A. fumigatus, A. niger, Penicillium citrinum, Alterneria* sp *and Mucor* sp against chromium at 100 ppm whereas, only *A. niger and Penicillium* sp tolerated at 500 ppm. Tolerance index (Ti) for fungal species were identified to be 73 % - 86% for *Aspergillus* sp., 65% for *Penicillium* sp. and less than 50% for other genera (**Figure 1**). Further secondary screening, with 1000 ppm chromium proved the bio sorption efficiency of *A. niger* to be 78 % for live and 62 % for dead fungal biomass. Accordingly, Price et al [18] have reported Aspergillus to be the most tolerating organism of chromium with a MIC of 400 mg/l, where Penicillium and Fusarium to be less tolerating (100 mg/l). Similarly, the chromate reductase activity of *Penicillium* and *A. niger* have been studied by Arévalo-Rangel et al [19] and Gu et al [20] respectively.

Effect of nutrients on biosorption

Effect of the environmental parameters, such as Carbon, Nitrogen and pH upon the microbial growth is illustrated for *A. niger* in (**Figure 2**), which shows much promising activity at pH 5 with malt and glucose as nutrient sources. This absorption may be due to the adaptations of fungal spores to germinate rapidly. Moreover, it indicates the ability of organisms at contaminated soils to possess amplified heavy metal absorption properties. In accordance with present study, Ezzouhri et al [16] has also confirmed the presence of similar organisms in heavy metal contaminated soils and also concluded that *Aspergillus* and *Penicillium* were capably tolerant to them in comparison to other organisms.

In present study, *A. niger* when supplemented with 1% glucose and malt extract at pH 5, showed maximum chromium removal of 82 % whereas, its minimal effect of 36 % was observed at constant pH 6, with peptone and mannitol as supplements. Furthermore, Rivastava and Thakur [21] suggested that the detoxification of

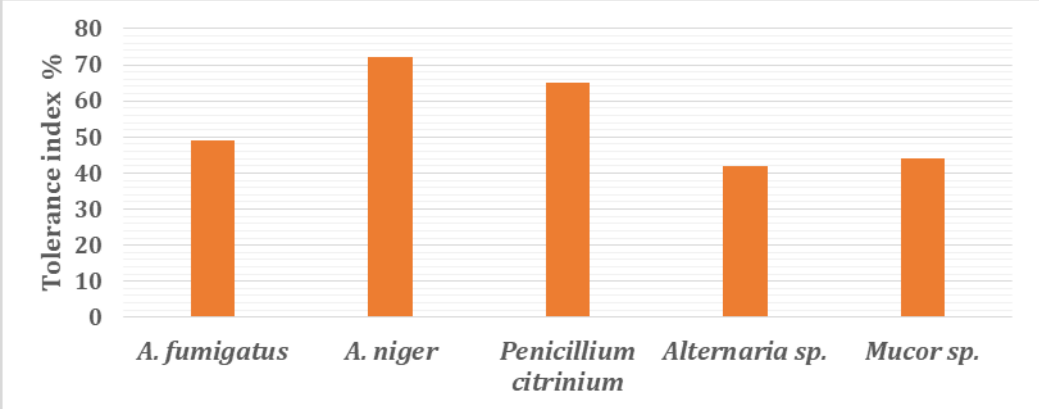

Figure 1 Tolerance index of fungal isolates.

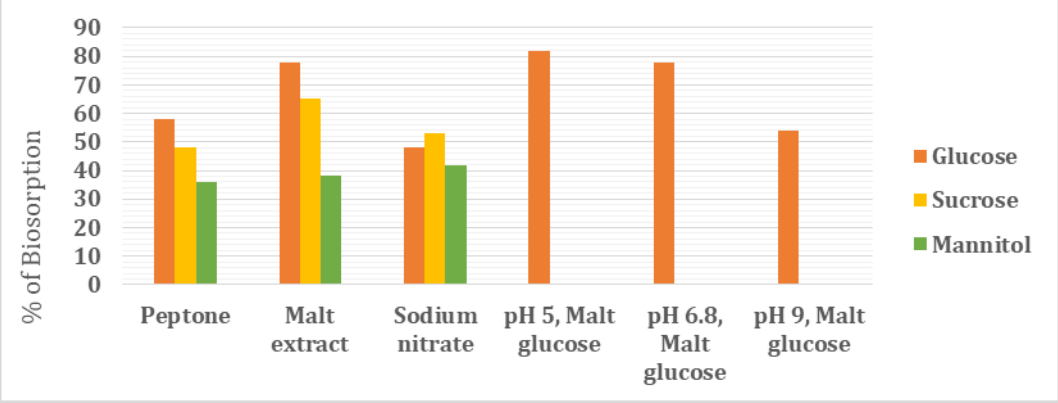

Figure 2 Effect of Carbon-Nitrogen and pH on Biosorption by *A. niger*.

chromium by *A. niger* may be mediated through certain enzymatic antioxidant systems such as peroxidase, catalase and ascorbate peroxide and also it removed more than 75 % chromium at pH 6 and 30°C.

Evolutionary relationships

18S rRNA gene of the most resourceful organism according to their gene sequences (**Table 3** and **Figure 3**) was found to be *A. niger* using BLAST analysis. *A. niger* (GenBank Accession Number KY354579) showed 99 % similarity to the existing organism. Further, Neighbor-Joining method inferred the evolutionary history by tree, using bootstrap consensus of 1500 replicates which represents the taxa and their relationship with ancestral origins. According to the bootstrap test, the percentage of their associated taxa is clustered next to the branches. Tree created, which is to scale represents the branch length to the evolutionary distance, by which the phylogenetic tree (**Figure 4**) is constructed.

Table 3. *A. niger* sequence.

AAGGTTCGTGCTTTCATCTAGAGCCCAACCTCCACCCGTGTTTACTGTASCTTAGTTGCTTCGGCGGGSCCGCCATTCAAGGMAGAMGGGGGCTCTGAGCCCC-GGGCCCGCGCCCGCCAGAGACACCACAAACTCTGTCTGATCTATGAAGTCTGAGTTATTGTTCCACAWTAYAATCAATGGATTCTTGTCGGATCGATAAAMGCCATTG-GCKAACCACTGCTAAYGCRATTMTTGATCTYTGTCTTAAAGCAAATGCTCCCTTGTTTTKGGGGRTTCTCGCCMAKTGTTTTTGCTATCATGGKTGGGGTTGGRAACC-SCCCTCGGAGYGCATCCACAAAGGRGSCMCCCGGAACCCTYTAWACATCTCTTAAC

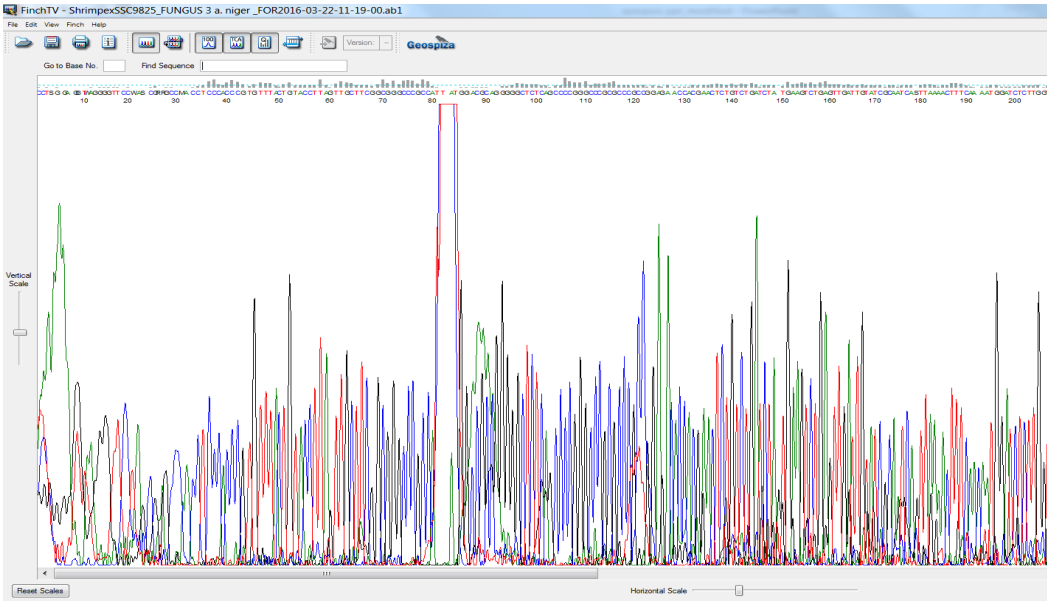

Figure 3 *A. niger* sequence.

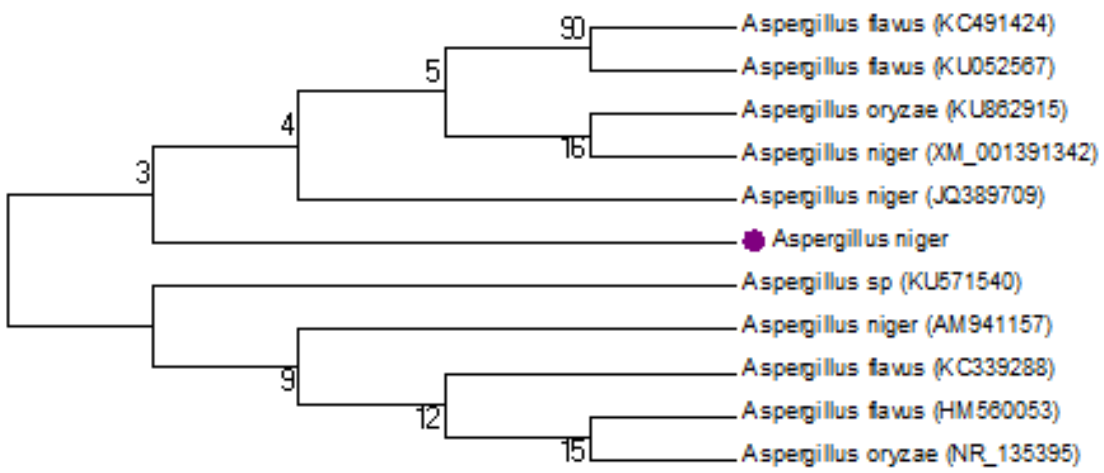

Figure 4 Phylogenetic tree based on 18S rRNA sequencing of isolated *A. niger*.

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Conclusion

Biosorption of heavy metals, especially chromium needs to be carried out proximately, to avoid the hazardous effects of these chemicals on the environment and also on all living organisms. Above all methods of effluent treatment, bio sorption using microbes are considered best due to their numerous advantages. On such an approach *Aspergillus niger* is identified as the best organisms to treat chromium. Additionally, their efficiency can be easily increased by maintaining their optimum parameters. Thereby, this study addresses a challenging concept.

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