ABSTRACT
The investigation focuses on the impact of Copper(II) and Zinc(II) ions on the growth, biosorption capacity and resistance mechanisms of Aspergillus spp. isolated from metallic industrial effluent. Heavy metal ions due to their high density are toxic to the living systems and hence when released into the environment cause substantial damage to the ecosystem. Bioremediation of heavy metal ions proves to be an exemplary method in reducing environmental pollution. The fungal isolate was screened for its ability to absorb heavy metal ions from nutrient medium supplemented with various concentrations of Copper(II) and Zinc(II) ions. Aspergillus spp. is adept in absorbing heavy metal ions from potato dextrose media bearing 100mg/L of Cu$^{2+}$ and 750mg/L of Zn$^{2+}$ ions. Addition of heavy metal ions in growth medium resulted in the substantial increase in the activities of copper-amine oxidase, Cu-Zn superoxide dismutase, catalase and peroxidase. Increased expression of copper-amine oxidase serves as an excellent indicator of intracellular reactive oxygen species (ROS) generation in fungal cultures under heavy metal stress. However the increased activities of Cu-Zn superoxide dismutase, catalase and peroxidase enable the mould to withstand the oxidative stress induced by heavy metal ions. These enzymes scavenge the ROS produced and allows Aspergillus spp. to grow past the toxic effects of Copper(II) and Zinc(II) ions. These increased enzymatic activities play a pivotal role in understanding the cellular and molecular abilities of Aspergillus spp., which can be effectively utilized in decreasing heavy metal pollution.
Keywords: Copper, Zinc, Bioremediation, Biosorption, *Aspergillus spp.*, Reactive oxygen species.

INTRODUCTION

Environmental pollution due to heavy metal ions is a major threat to the ecosystem. With rapid industrialization, heavy metals are being discharged into the environment in greater amounts. Chemical and metallurgical manufacturing units are principle sources of heavy metal contamination.[1] Heavy metals like copper, zinc, lead, arsenic, mercury and chromium are most commonly involved in environmental pollution. The heavy metals are highly toxic even in low concentrations and become a severe threat to living organisms due to the natural biomagnification processes. Conventional physical and chemical techniques like ion-exchange, precipitation, membrane technologies, electrochemical treatments are generally used in the removal of heavy metal ions from aqueous solutions.[2] However these methods are ineffective in removing low concentrations of heavy metal ions. Under such circumstances bisorption of heavy metal ions serves as an effective technique in reducing environmental pollution.

Microbial communities play a pivotal role in the removal of heavy metal ions from industrialized and polluted areas. Bioremediation by microorganisms is advantageous because of its effectiveness in a broad spectrum of conditions like varying pH and temperature.[3] They are effective in eliminating heavy metal ions from contaminated sources as they can influence the activity and mobility of metals through reduction, accumulation, mobilization and immobilization.

Filamentous fungi are skilled in accumulating heavy metal ions from contaminated sources by means of biological and physico-chemical mechanisms. Price et. al(2001) evaluated fungi for the purpose of heavy metal removal and found *Aspergillus spp.* to be best suited. Chitin, chitosan,[4] β-glucan present in the cell wall of *Aspergillus* are potential binding sites of heavy metal ions. Binding of the metal ions to these cell wall components allows its immobilization and subsequent accumulation.[5]

The present study was carried out to evaluate the ability of *Aspergillus spp.* to remove copper and zinc ions found in the effluents from various industries. This investigation was also conducted to understand the toxic effects of heavy metals on the mould and the processes by
which it withstands such detrimental effects. It is important to comprehend the resistance mechanisms of *Aspergillus spp.* as the rate of metal removal directly depends on the efficiency of such mechanisms.

**MATERIALS AND METHODS**

1. **Sources and collection**
   The effluent was collected in a sterilized 250 ml Erlenmeyer flask from a metallic industry in Cossipore, West Bengal.

2. **Characterization of sample**
   i) **Physical Characterization**
   A. **Determination of Total Dissolved Solid (TDS) of the sample:** Whatman no. 1 filter paper disc was dried at 90°C for 10 minutes in a hot air oven and subsequently the dry weight was measured. The dried filter paper disc was used to filter out 10 ml of the effluent sample. The filter paper along with the adhered residue was dried at 90°C for 10 minutes and the dry weight was again measured. The process was repeated thrice.

   B. **Determination of Electrical Conductivity (EC) and Total Suspended Solid (TSS) of the sample:** 50 ml of the effluent sample was filtered using Whatman no. 1 filter paper and the EC and TDS were determined at 25°C using a standard water analyzer.

   ii) **Chemical Characterization:**
   A. **Determination of pH of the effluent sample:** The effluent sample was filtered using Whatman no. 1 filter paper and the pH was subsequently determined using a standardized pH meter.

   B. **Quantification of the amount of Cu(II) and Zn(II) in the sample:** To quantify the amount of copper and zinc present, the sample was treated with 33% concentrated nitric acid in 1:1 ratio and heated in a crucible on a hot plate for 5 minutes. The digested sample was filtered using Whatman no. 1 filter paper and necessary volume makeup was done using double distilled water. This digested sample was utilized to determine the concentration of copper and zinc present by ICP-MS (Inductively Coupled Plasma-Mass Spectrometry).

3. **Isolation of fungus from industrial effluent:** The sample was collected in sterilized Erlenmeyer flask from Cossipore, West Bengal and examined within 24 hours. Isolation of fungi was achieved by employing serial dilution technique. The water sample was serially
diluted (10-10,000 folds). Aliquots of 100µl from each dilution were plated on Potato Dextrose Agar (PDA) (containing 20g/L of sucrose and 200g/L of potato) by employing pour plate technique. The PDA plates were incubated at 25°C for 4 days to ensure proper growth. Purified fungal growth was obtained by repeated inoculation on PDA plates.

4. Identification of the isolated fungus:- Pure cultures of the isolated fungus were examined and identified based on their colony morphology and microscopic characteristics.

Small amount of fungal sample was placed on a drop of lacto-phenol cotton blue stain, teased adequately and then visualized under microscope.

Using the keys of Ellis et.al (1971),[6] Ellis et.al (1976),[7] and Joseph C. Gilman (1998),[8] the pure culture was characterized to the genus level. The pure culture was identified as a member of the genus *Aspergillus*.

5. Biomass reduction assay:- The purified fungal isolate was subjected to heavy metal stress. 8mm agar plugs of pre-grown fungal isolate were added to 50 ml of Potato Dextrose Broth (PDB) in 100 ml conical flasks supplemented with different concentrations of copper and zinc ions.[9] The metal salts used were copper sulfate and zinc sulfate. A control was prepared where the inoculated PDB was devoid of any heavy metal solution. The inoculated flasks were incubated at 25°C for 10 days. After the destined incubation period, the fungal mat developed on the surface of the broth was filtered out and dried at 90°C for 1 hour. The weight of the dried biomass was measured and the reduction in the fungal biomass in presence of metal solutions was determined by comparing with the control.

The median lethal dose (LD 50), is the concentration of metal ion that kills half the members of a tested population after a specified test duration. By comparing the dry weight of the fungus (growing in presence of metal) with the control, the LD 50 of copper and zinc ions for *Aspergillus* spp. was determined.

6. Quantification of metal ion uptake by *Aspergillus* spp.:-

Due to toxic effects of copper and zinc, the fungal biomass decreased with increasing concentrations of heavy metal ions. The biomass from heavy metal solution flasks which depicted LD 50 were treated with 33% nitric acid. The acid-treated biomass was utilized to quantify the amount of copper and zinc ions absorbed by the fungus, adopting the technique of ICP-MS.
In this technique sample solutions are introduced into the ICP as an aerosol that is carried into the center of the plasma (superheated inert gas). The plasma desolvates the aerosol into a solid, vaporizes the solid into a gas, and then dissociates the individual molecules into atoms. This high temperature source (plasma) excites the atoms and ions to emit light at particular wavelengths, which correspond to different elements in the sample solution. The intensity of the emission corresponds to the concentration of the element detected.\[10]\]

7. Enzyme assay:- The culture filtrate obtained from flasks which showed LD 50 were utilized to estimate the activity of copper-amine oxidase, Cu-Zn superoxide dismutase, catalase, and peroxidase. The activity of these enzymes under heavy metal stress was compared with their respective activity in the control setup.

i) Copper Amine Oxidase (E.C 1.4.3.6) assay:- In the presence of suitable amine substrates, amine oxidase enzymes generate $\text{H}_2\text{O}_2$ which then derives peroxidase dependent reaction of 4-aminoantipyrine. A subsequent interaction with vanillic acid generates stoichiometric amounts of quinoneimine dye, the appearance of which is monitored at 498nm.

ii) Copper-Zinc Superoxide Dismutase (E.C 1.15.1.1) assay:- SOD was extracted taking the culture filtrate. Salting out of the appropriate protein fraction was done by making the filtrate to 30-90% (NH$_4$)$_2$SO$_4$ (w/v). The precipitate thus obtained was dissolved in 0.5ml of extraction buffer (without $\beta$-ME) and used as the crude enzyme extract. Crude extracts from all the samples were assayed for SOD activity spectrophotometrically using the assay system consisting of methionine, riboflavin, nitroblutetrazolium (NBT) as described by Beaucchamp and Fridovich in 1971.\[11]\] The absorbance was taken at 632.8nm spectrophotometrically.

iii) Catalase (E.C 1.11.1.6) assay:- The culture filtrate was taken for catalase assay. Catalase was assayed according to the modified method of Chance and Maehly, 1955.\[12]\] 3ml of reaction mixture contained 50mM phosphate buffer (pH 7.0), 15mM $\text{H}_2\text{O}_2$ and 75$\mu$g of crude enzyme extract. The decrease in $\text{H}_2\text{O}_2$ was followed as decline in absorbance at A=240nm measured spectrophotometrically. The catalase was expressed in units where 1 unit of CAT converts 1$\mu$mol of $\text{H}_2\text{O}_2$ equivalence/ min/ mg of total protein.
iv) **Peroxidase (E.C 1.11.1.7) assay:** 3ml of pyrogallol solution and 0.1ml of prepared extract were taken in a cuvette. The optical density (O.D.) of the reaction mixture was measured at 430nm and was taken as control. This mixture was taken as the blank and later 0.5ml of 1% H₂O₂ was added and mixed thoroughly. The O.D was measured at regular intervals at 430nm.

**RESULTS**

1. Characteristics of sample

i) Physical characteristics

A. Electrical Conductivity and Total Dissolved Solid of the sample

**Table 1- Determination of EC and TSS of the effluent sample**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Electrical conductivity(µS)</th>
<th>Total Suspended Solid(ppt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effluent from metallic industry in Cossipore, West Bengal.</td>
<td>88.46</td>
<td>35.14</td>
</tr>
</tbody>
</table>

B. Total Suspended Solid of the sample

**Table 2- Determination of TDS of the effluent sample**

<table>
<thead>
<tr>
<th>No. of observation</th>
<th>Dry weight of the filter paper (gms.) [A]</th>
<th>Dry weight of the paper and adhered residue (gms.) [B]</th>
<th>Difference in weight (gms.) [B-A]</th>
<th>Amount of sample used (ml)</th>
<th>TDS (gm/L) ((B - A) \times 100/10)</th>
<th>Mean TDS (gm/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.625</td>
<td>0.789</td>
<td>0.164</td>
<td>10</td>
<td>164</td>
<td>179.6±</td>
</tr>
<tr>
<td>2.</td>
<td>0.527</td>
<td>0.727</td>
<td>0.200</td>
<td>200</td>
<td>175</td>
<td>10.65</td>
</tr>
<tr>
<td>3.</td>
<td>0.454</td>
<td>0.629</td>
<td>0.175</td>
<td>175</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ii) Chemical characteristics

A. **pH of the sample:** The pH of the effluent was found to be 2.3, representing the acidic nature of the sample.

B. **Concentration of Cu(II) and Zn(II) in the sample:** The ICP-MS (Inductively Coupled Plasma- Mass Spectrometry) study on the effluent from metallic industry revealed a copper concentration of 4233mg/L and a zinc concentration of 2623mg/L.
Table 3: Determination of concentration of Cu and Zn present in the industrial effluent

<table>
<thead>
<tr>
<th>Sample</th>
<th>Heavy metal estimated</th>
<th>Method</th>
<th>Result</th>
<th>Limit Reporting</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effluent from metallic industry</td>
<td>Copper</td>
<td>APHA 22nd. EDN.:2012-3120 B</td>
<td>4233</td>
<td>0.01</td>
<td>mg/L</td>
</tr>
<tr>
<td></td>
<td>Zinc</td>
<td></td>
<td>2623</td>
<td>0.01</td>
<td>mg/L</td>
</tr>
</tbody>
</table>

2. Identification of the fungal isolate- The fungal isolate was identified by studying its colony characteristics and microscopic features.

Table 4- Colony morphology

<table>
<thead>
<tr>
<th>Colour</th>
<th>Reverse colour</th>
<th>Size</th>
<th>Texture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green</td>
<td>Hyaline</td>
<td>6.5-7 cm (after 5 days)</td>
<td>Granulated</td>
</tr>
</tbody>
</table>

Table 4- Microscopic characteristics

<table>
<thead>
<tr>
<th>Vesicle</th>
<th>Conidia</th>
<th>Conidial surface</th>
<th>Hyphae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large and nearly</td>
<td>Small ,attached to the</td>
<td>Smooth</td>
<td>Long, septate, unbranched and hyaline</td>
</tr>
<tr>
<td>circular</td>
<td>vesicle forming radial chains</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. Biomass reduction assay: - The reduction in the biomass was observed with the increase in concentration of zinc and copper ions. Toxic effects of heavy metals have a direct impact on fungal growth which is manifested as the reduction in the biomass. The median lethal dose (LD 50) of copper for Aspergillus spp. is 100mg/L (Fig.2) and that of zinc is 750mg/L (Fig.3). This study shows that copper is more toxic to the proliferation of the fungal cells than zinc.

![Fig. 1- Growth of Aspergillus spp. in control](image-url)
Fig. 2- Growth of *Aspergillus* spp. In presence of 100mg/L of Cu(II)

Fig. 3- Growth of *Aspergillus* spp. in presence of 750mg/L of Zn(II)

4. **Quantification of heavy metal uptake by *Aspergillus*:-** ICP-MS study on the fungal biomass revealed the biosorption of heavy metals by *Aspergillus* spp. The mould is adept in internalizing heavy metal ions from culture media, thus proving to be effective in bioremediation. Amount of zinc absorbed by *Aspergillus* spp. is greater than that of copper, reiterating the greater toxic effects of copper over zinc. The amount of metal absorbed by the mould can be calculated using the following formula

\[ Q = (C_f - C_i) \times \frac{V}{m} \]

where,

- \(Q\) = mg of metal absorbed per gram of biomass,
- \(C_f\) = concentration of metal ion present in fungal biomass under test setup (mg/L)
- \(C_i\) = concentration of metal ion present in fungal biomass under control setup (mg/L)
- \(V\) = volume (L) of the reaction mixture containing metal ions
m= dry weight of biomass in reaction mixture containing metal ions (gm.).[13,modified]

Instead of measuring the amount of metal ion left in the reaction mixture, the amount of metal ion present in the developed biomass has been quantified.

**Table 5: Biomass reduction with increasing concentrations of Cu$^{+2}$ and Zn$^{+2}$ ion**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight of Aluminium foil (in grams) [A]</th>
<th>Weight of Aluminium foil + fungal biomass (in grams) [B]</th>
<th>Fungal biomass/ Dry weight (in grams) [B-A]</th>
<th>Percentage reduction in biomass X 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus spp. in control setup</td>
<td>0.432</td>
<td>0.925</td>
<td>0.493</td>
<td>–</td>
</tr>
<tr>
<td>Aspergillus spp. in presence of 50mg/L of Cu$^{+2}$</td>
<td>0.488</td>
<td>0.847</td>
<td>0.359</td>
<td>27.18</td>
</tr>
<tr>
<td>Aspergillus spp. in presence of 100mg/L of Cu$^{+2}$</td>
<td>0.418</td>
<td>0.671</td>
<td>0.253</td>
<td>48.68</td>
</tr>
<tr>
<td>Aspergillus spp. in presence of 150mg/L of Cu$^{+2}$</td>
<td>0.389</td>
<td>0.44</td>
<td>0.051</td>
<td>89.65</td>
</tr>
<tr>
<td>Aspergillus spp. in presence of 250mg/L of Zn$^{+2}$</td>
<td>0.453</td>
<td>0.996</td>
<td>0.443</td>
<td>10.14</td>
</tr>
<tr>
<td>Aspergillus spp. in presence of 500mg/L of Zn$^{+2}$</td>
<td>0.514</td>
<td>0.858</td>
<td>0.339</td>
<td>31.23</td>
</tr>
<tr>
<td>Aspergillus. spp in presence of 750mg/L of Zn$^{+2}$</td>
<td>0.442</td>
<td>0.704</td>
<td>0.262</td>
<td>46.85</td>
</tr>
</tbody>
</table>

**Table 6: Determination of concentration of Cu and Zn present in the fungal biomass under different growth conditions**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Heavy metal estimated</th>
<th>Method</th>
<th>Result</th>
<th>Limit of Reporting</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass formed in metal devoid conditions (Control)</td>
<td>Copper</td>
<td>APHA 22nd. EDN.:2012-3120 B</td>
<td>6.65</td>
<td>0.01</td>
<td>mg/L</td>
</tr>
<tr>
<td></td>
<td>Zinc</td>
<td></td>
<td>22.96</td>
<td>0.01</td>
<td>mg/L</td>
</tr>
<tr>
<td>Biomass formed in presence of 100 mg/L of Cu$^{+2}$ ions (Test 1)</td>
<td>Copper</td>
<td></td>
<td>83.75</td>
<td>0.01</td>
<td>mg/L</td>
</tr>
<tr>
<td>Biomass formed in presence of 750 mg/L of Zn$^{+2}$ ions (Test 2)</td>
<td>Zinc</td>
<td></td>
<td>660.75</td>
<td>0.01</td>
<td>mg/L</td>
</tr>
</tbody>
</table>
Table 7: Amount of Cu and Zn absorbed by Aspergillus spp. from reaction mixture

<table>
<thead>
<tr>
<th>Sample</th>
<th>Heavy metal absorbed</th>
<th>Concentration of heavy metal ion in control [C_i] (in mg/L)</th>
<th>Concentration of heavy metal ion in test [C_f] (in mg/L)</th>
<th>Volume [L] of the reaction mixture (in litres)</th>
<th>Dry weight of biomass in the reaction mixture [m] (in grams)</th>
<th>Metal ions (mg) biosorbed per gram of biomass [Q]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass formed in presence of 100 mg/L of Cu^{2+} ions (Test 1)</td>
<td>Copper</td>
<td>6.65</td>
<td>83.75</td>
<td>0.050</td>
<td>0.253</td>
<td>15.23</td>
</tr>
<tr>
<td>Biomass formed in presence of 750 mg/L of Zn^{2+} ions (Test 2)</td>
<td>Zinc</td>
<td>22.96</td>
<td>660.75</td>
<td></td>
<td>0.262</td>
<td>121.71</td>
</tr>
</tbody>
</table>

5. Enzyme assay: - Aspergillus spp. is able to absorb copper and zinc from the environment. However it has to combat the toxic effects of these heavy metals inorder to enhance its bioremediation potential. The following enzymatic assays revealed the cause for such toxic effects and the ability of Aspergillus spp. to withstand such heavy metal stress.

i) Copper-Amine Oxidase assay analysis: - This assay revealed the increased activity of copper-amine oxidase in presence of copper and zinc ions. This increased activity of copper-amine oxidase thus serves as an indicator for the heavy metal ion mediated generation of Reactive Oxygen Species (ROS) in growing cell cultures, thus imparting oxidative stress to the mould. (Fig. 4)

ii) Analysis of ROS scavenging enzymes: - The assay for SOD, catalase and peroxidase revealed their increased activities in the culture filtrate in presence of copper and zinc ions. Enhancement in the enzymatic activities manifests the ability of Aspergillus spp. to withstand oxidative stress owing to the heavy metal ion mediated ROS generation.

(Fig. 5, 6, 7) Graphs: - In figures 4 to 7 Sample A refers to Aspergillus spp. growing in control setup, Sample B refers to Aspergillus spp. growing in presence of 100mg/L of Cu^{2+} ions and Sample C refers to Aspergillus spp. growing in presence of 750mg/L of Zn^{2+} ions.
**Fig. 4:** Effect of increase in heavy metal ions on copper-amine oxidase activity

**Fig. 5:** Effect of increase in heavy metal ions on SOD activity

**Fig. 6:** Effect of increase in heavy metal ions on catalase activity
4. DISCUSSIONS

Heavy metal ions present in the natural and industrial areas are strong pollutants. Many conventional techniques are employed for their detoxification (e.g. membrane separation, ion exchange, neutralization, precipitation). However these techniques are ineffective in removing low concentrations of heavy metal ions. Biosorption using microbial biomass as bioabsorbent is an extremely effective technique, capable of removing low concentrations of heavy metal ions.\cite{14}

In this present study, filamentous fungal isolate of *Aspergillus* spp., isolated from industrial effluent was employed to evaluate its ability to absorb heavy metal ions. The fungal isolate was able to grow at copper concentrations ranging from 50mg/L to 150 mg/L. Price et.al(2000) reported that *Aspergillus niger* was able to remove 91% copper from swine wastewater. They reported that the fungi is able to grow on plates amended with a level of Cu, fives time greater than that inhibitory to the growth of *Saccharomyces cerevisiae*.\cite{15} Jayram et. al (2014) reported that *Aspergillus flavus* shows maximum removal of 25.9mg/gm of bioabsorbent when concentration of Cu(II) is maintained at 0.1 mg/L.\cite{16}

Zinc is an essential element required by all microorganisms; however at high concentration it can be toxic. *Aspergillus* spp. was able to grow and proliferate at Zn concentrations ranging from 250mg/L to 750mg/L. Price et. al (2000) reported that *Aspergillus niger* is capable of removing 70% of zinc from treated swine effluent. Faryal et. al(2000) showed that *Aspergillus fumigatus* RH05 showed 65% absorption at 40⁰C after 30 minutes of incubation.\cite{17}
The present study also reveals the effect of Cu(II) and Zn(II) on the activities of copper-amine oxidase, Cu-Zn superoxide dismutase, catalase and peroxidase. Copper-amine oxidase catalyze the oxidation of primary amines to aldehydes with the subsequent production of ammonia and hydrogen peroxide.\[18]\] Increase in the activity of the enzyme in response to 100mg/L of Cu(II) and 750mg/L of Zn(II) indicates the heightened generation of reactive oxygen species (ROS), detrimental to the survivability of the mould. However the enhanced production of Cu-Zn SOD, catalase, peroxidase denotes the antioxidant response against stress induced by heavy metal ions. Luna et. al (2013) reported that Aspergillus niger exhibited increased activity of catalase, oxidase, peroxidase in response to increased concentration of Cu(II) ions.\[19]\]

CONCLUSION
Metals such as copper and zinc are required in various biological activities; however they exhibit toxicity at certain levels. Our findings indicate that the mould Aspergillus spp., isolated from heavy metal contaminated sources is tolerant to high concentrations of heavy metal ions and is highly capable of absorbing the same, thus decreasing environmental pollution. Oxidative stress induced by heavy metal ions is overcome by the mould’s antioxidant response which increases the potential of bioremediation substantially.

ACKNOWLEDGEMENT
We would like to thank Rev. Fr. Dr. J. Felix Raj, S.J., principal, St. Xavier’s College, Kolkata for his blessings and the Department of Microbiology, St. Xavier’s College, Kolkata, for their support. We are indebted to Dr. Sudeshna Shyam Chowdhury for her constant support and encouragement.

REFERENCES


