Bioremediation of Lead (Pb) by using *Aspergillus terreus* Isolated from Polluted Soil

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Abstract: Industrial effluent causes serious problem to the environment, it contain toxic chemicals such as heavy metals, dyes and other substances which affect water bodies, soil and other parts of the ecosystem. Heavy metals such as Lead (Pb), Cadmium (Cd), Chromium (Cr) and Copper (Cu) are very hazardous to human life, when released through human anthropogenic activities. Methods of removal by physicochemical techniques were found inadequate. Bioremediation procedures can be employed to remove waste products from the environment which is eco-friendly and less cost-effective. Pb-resistant fungus was isolated from the polluted soil. It was grown on Sabouraud Dextrose Broth (SDB) by enrichment method and plated on Sabouraud Dextrose Agar (SDA) and then screened at various concentrations of Pb ions concentrations up to 700mg/L. The 18S rRNA was carried out and based on that Clustal X software was used to identify the isolate as *Aspergillus terreus*. The uptake of the Pb ions was also carried out and enhanced in the liquid medium by optimization of the conditions which includes pH, temperature, inoculums size and incubation time. The highest Pb removal was found to be 84.63%, with dried biomass of 1.78 mg. Scanning Electron Microscopy revealed the structure of the isolates before and after the treatment with Lead heavy metal. The isolate could be used for the removal of contaminants especially hazardous chemicals and heavy metals from the environment.
Key words: *Aspergillus terreus*, Bioremediation, Heavy metal, Lead, Toxic.

INTRODUCTION

Industrialization leads to the rapid increase in production in many areas, which in other hand causes anthropogenic impact to the environment due to some activities carried out in the environment, release of hazardous chemicals and heavy metals affect the essential parts of the ecosystem. Most industries release their effluents which contain heavy metals higher than permissible limit by the World Health Organization (Wagner, 1993). As such the environment turn to be dumping ground for deposition of industrial wastes beyond imagination, which cause serious threat to farm land, water bodies and human life. It was also reported that heavy metals in the underground water have caused various diseases in human beings and also affect metabolic functions negatively (Bernard *et al*., 1992; Ademorotti *et al*., 1992; Umar *et al*., 2014).

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Physicochemical methods of removing heavy metals such as filtration, ion exchange, chemical precipitation electrochemical treatment, membrane technologies, adsorption on activated carbon and evaporation are found ineffective, difficult and very costly. Also these methods cannot be carried out at large scale (Negri and Hinchman, 1996).

The past two decades have seen a tremendous upsurge in the search for cost effective and environmentally friendly, alternatives to the conventional method for dealing with wastes. The technologies that have emerged as most promising are those that closely mimics the time tested, natural system that have restored environments to their original status following undesirable perturbation. Of all the technologies that have been investigated, bioremediation has emerged as the most desirable approach for cleaning up many environmental pollutants in effluent (Brierky, 1990; Tobin *et al*., 1994; Leusch *et al*., 1995 and Chaudhry *et al*., 1999).

Bioremediation is a pollution control technology that uses biological system to catalyze the degradation of or transformation of various toxic chemicals to less harmful forms (Diaz, 2008).

The general approaches to bioremediations are to enhance natural biodegradation by natural organisms (Intrinsic bioremediation), to carry out environment modification by applying nutrients or aeration with oxygen (biostimulation), or though addition of micro-organisms (bioaugmentation) (Lowerence, 2002). The ability of microorganisms to transform a variety of chemicals has led to their use in bioremediation process. It is now an exact science and includes processes such as intrinsic bioremediation, biostimulation and bioaugmentation (Atlas and Unterman, 1999). Intrinsic bioremediation is the process whereby natural indigenous micro flora and environmental conditions interact to bring about the natural attenuation of pollution to safe levels, within an acceptable time frame (Elshanshoury *et al*,. 2012).

MATERIALS AND METHODS

Sample Collection and Isolation

The soil samples were collected from the soils contaminated with industrial effluent at Challawa Industrial Estate Kano, Kano State, Nigeria. The samples were obtained from contaminated site few inches below the soil surface and transported to the Laboratory. one gram of the soil sample was weighed and put in the 9 mL of sterilized distilled water and four folded serial dilution was carried out. Stock solution of $Pb(NO_3)_2$ was prepared and then working solutions of Pb ion. One militre of four folded serial dilution was put into sterilized 100 mg/L of Pb-ion concentration working solution, enriched with Sabouraud Dextrose Broth (SDB) in the 250 mL Erlenmeyer flask and placed in the orbital shaker at 100 rpm for 24 h as described by (Lee *et al*., 2012). Then 1 mL of the aliquot was taken and inoculated on the Sabouraud Dextrose Agar by spread method and incubated for 96 h (Clausen *et al*., 2000).

Screening of Pb Resistant isolate and Identification

Different concentration of 100 mL of Pb-ion concentrations were prepared of (100, 200, 300, 400, 500 and 700 mg/L) were put into 250 mL of Erlenmeyer flasks enriched with Sabouraud Dextrose Agar (SDA), which was sterilized at 15 lbs/psi, then poured unto the plates and allowed to solidify. The fungal isolate obtained was inoculated on the plates; control was made which were then incubated for 96 h. The resistant isolates obtained were that grow on the plates at different concentrations, the isolate that was able to grow at high concentrations of Pb was selected as described by Price *et al*., (2001). The resistant fungal isolate that grown at high concentrations of Pb was selected. Lactophenol blue cotton staining was carried out for preliminary study of the morphological features of the fungal isolate.

DNA Extraction

DNA was extracted from the fungal isolate using a commercial Nucleospin DNA extraction kit according to the Manufacturer's instruction (Clontech CA, USA). The sample was transferred into 1.5 mL microcentifuge tube containing 0.5 mm diameter glass beads and 0.2% SDS (Sodium dodecyl sulphate). Cell destruction was achieved by vortexing in the microcentrifuge tube for 15 min. Six hundred microliters of Sorbitol buffer containing 1M Sorbitol, 100 mM EDTA, 14 mM β – Mercaptoethanol and 10 µL of lyticase (Sigma Missouri, USA) was added and incubated at 30^0C for 30 min. The mixture was then centrifuged at 6000 rpm for 10 min. The spheroplast pellets were suspended in lyses buffer (Nucleospin kit) and 25 µL of proteinase K (20 mg/mL) was added and mixed by vortexing and incubated at 56^0C over night. The sample was also centrifuged at 10, 000 rpm for 5 min. The supernatant was transferred to a fresh 1.5 microcentrifuge tube and kept at -20^0C.

Primer Design

The conserved region 18S rRNA gene for fungal isolate was targeted for the study. From Gen Bank data base sequences of the fungal isolates which were very close to the sample were obtained, based on that unique primers were designed to Genus level.

The forward and reverse primers of fungal isolate: TCTTTATGGCCACCTCCCA and AATCACCCACCGACCTTA respectively. To verify the specificity of the designed primers, the BLAST Program at National Center for Biotechnology Information (NCBI) website was employed to search the primer near exact sequences. Primer for the isolate was manufactured by Macrogene Company, Seoul, South Korea.

Polymerase Chain Reaction

The polymerase chain reactions were performed in 20 µL micro tubes containing 2 µL of the genomic DNA sample, 750 mM TRIS – HCl buffer (pH 8.8 at 25^0C), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20; 2.5 mM $MgCl_2$; 0.16 mM dNTP mix; 20 pmol of forward and reverse primers and 0.75 µL Taq DNA polymerase (Macrogene Company, Seoul, South Korea). Amplification was carried out in the thermal cycler (Eppendorf Mastercycle 5330) with initial denaturation at 95^0C for 4 min, followed by 30 cyles of denaturation at 95^0C for 30 sec, annealing at 55^0C. The thermal cycles were terminated by final extension for 5 min at 72^0C. The analyses of PCR products were performed on 1% agarose gel. The molecular weight markers 100 bp ladder (Fermentas, Lithuania) was used and the gel was run at 100 V for 45 min at room temperature. The PCR products were stained with Ethidium bomide and visualized by image analyser (Chemilmage 550, Alpha Intech, CA, USA).

Also, the PCR product was sequenced using an automated DNA sequencer at Macrogene Company (Seoul, South Korea) and based on the sequences obtained, 18S rRNA for the fungal isolate phylogenetic tree was constructed using Clustal X software (Tamura, 2011). The resistant fungal isolate was identified *Aspergillus terreus*. The isolate was submitted to the National Center for Biotechnology Information, USA. The accession number assigned to the isolate was KY50723.

Determination of Bioremoval of Pb from Liquid Medium

Concentration of Pb ion of 100 mg/L was enriched with SDB medium volume 100 mL in the 250 mL Erlenmeyer flask and sterilized. The culture of *A. terreus* was grown on SDA to the end of exponential phase, in which sterilized distilled water was poured into the test tube and vertex for 2 min and 1 mL was taken, the spores were counted in the haemocytometer to be 1.6 x 10^7 spores/mL then dispensed into the flask and placed in the orbital shaker at 30^0C under 100 rpm for 96 h. Control was made without inoculation of the fungal spores and kept under the same condition, as described by Clausen *et al.,* (2000). The fungal biomass grown were filtered with No1 Whattman filter paper and acidified with 1ml of 95% H_2SO_4. The residual of Pb was determined by Atomic Absorption Spectrophotometer (model GBC 932) as reported by Abou Zeid *et al*., (2009). The percentage of Pb removal of biomass was according to the equation of Valesky and May-Phillips (1995).

$$\text{Percentage of Removal} = \frac{\text{Initial concentration- Final concentration}}{\text{Initial concentration}} \times 100$$

Optimization of Bioremoval of Pb under different Conditions in the Liquid Medium

Effect of different pH Values

Different pH values (4, 5, 6, 7 and 8) of 100 mg/L of Pb concentrations containing Sabouraud Dextrose containing were taken 100 mL of flask. Sterilized and inoculated with mL suspension of spores of *Aspergillus terreus* at 30^0C in the orbital shaker under 100 rpm for 96 h.

Effect of different Incubation Temperature

One hundred milliliter of SDB having 100 mg/L of Pb solution was adjusted to pH 6, sterilized and inoculated with 1 mL of suspension of the spores into the flask and incubated at different temperature conditions (25, 30, 35 and 40 ^{0}C) in the orbital shaker at 100 rpm for 96 h.

Effects of Inoculums size and Incubation Time

The medium solutions of SDB containing 100 mg/L of Pb were made, sterilized and inoculated with different inoculums sizes of *Aspergillus terreus* suspensions (2, 4, 6, 8 and 10 ml) and kept at 30^0C and pH 6 in orbital shaker at 100 rpm for 96 h. The set up were made with inoculums size of 8 mL of *A. terreus* for the incubation periods (72, 96, 120 and 144 h) at 100 rpm at 30^0C. The fungal biomass obtained was filtered with Whatman filter paper No. 1, then dried it in the oven and weight.

Preparation of sample for Scanning Electron Microscopy (SEM)

Ten milliliter of the sample of the broth suspension culture of resistant fungal isolate with Pb from flask was centrifuged at 8,000 rpm at 4^0C for 5 min. The cells were washed twice with 0.1 M Phosphate buffered saline (PBS; 15 mM phosphate buffer, 138 mM NaCl, 2.7 Mm KCl, pH 7.4 at room temperature) and fixed over night in 2% glutaraldehyde (prepared in 0.1 M PBS). The cells were washed with PBS, distilled water then with ethanol series (10% to absolute) for 30 min at each concentration. Samples were placed on brass stub, sputter–coated with gold.SEM analysis (SEM–JEOL–JSM5800LV) was carried out to detect Pb that was either absorbed or entrapped in the cell wall of the resistant fungal isolate Walsh and Halloran, (1996).

RESULTS AND DISCUSSION

The fungal specie isolated from the soil sample collected by enrichment method was screened at various concentrations of Pb up to 700 mg/L. It was found to be tolerant to that concentration. Based on 18S rRNA sequences and Clustal X analysis the resistant isolate fungal specie was identified as *Aspergillus terreus* (Fig. 1). One hundred milligram per litre of Pb concentration in the liquid containing SDB medium was used to determine the bioremoval of Pb which was found to remove 74.28% of Pb; this corresponds to the findings of (Abou Zeid *et al*., 2009).

The optimizations of bioremoval of Pb under different conditions (pH, temperature and inoculums size and incubation time) were studied. Different pH values were taken (4, 5, 6, 7 and the maximum removal was obtained at pH 6 with percentage of removal of 75.61%. The bioremoval of Pb from the liquid medium started to increased significantly with the increase of pH, it produced higher activity at pH 6, and then decreased at pH value of 7. This correspond with (Quresh, 2003) that the biosorption of *Aspergillus* sp. increased significantly with increase of pH 2 to 6 because of osmotic concentration. For the temperature the maximum removal was observed at 30^0C with value of 78.40% then the activity started to come down at 35^0C and 40^0C respectively. Also similar to the work of Strandbergy *et al*., (1981) that demonstrated the optimum temperature of uptake of Uranium contaminant increases with the temperature in the range 20 – 30^0C and decreased at higher temperature because of its effect on integrity of the cell membranes and prevent compartmentalization of metal ions, which lead to reduction in the removal activity.

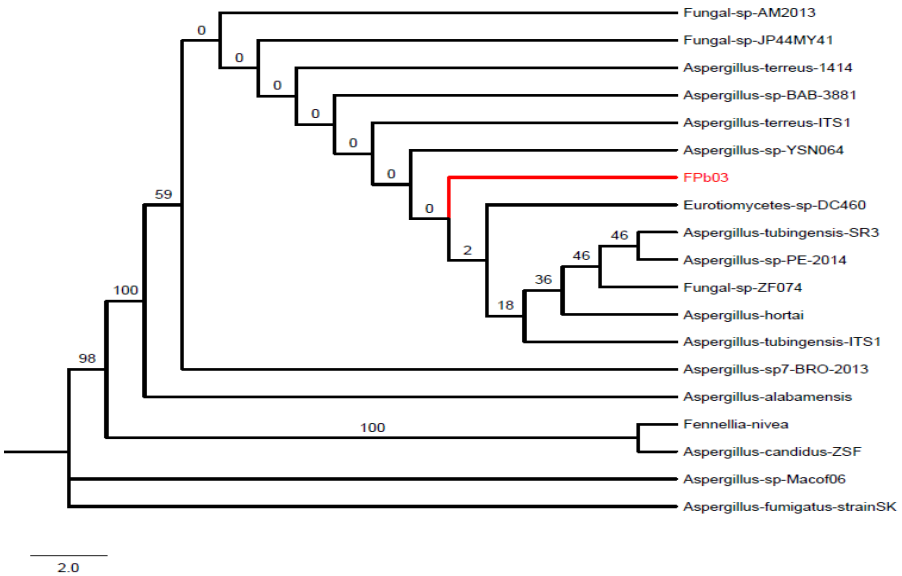

Figure 1: Neighboring-joining tree showing phylogenetic relationship based on the 18S rRNA gene sequences have shown to be closely related with *Aspergillus terreus*.

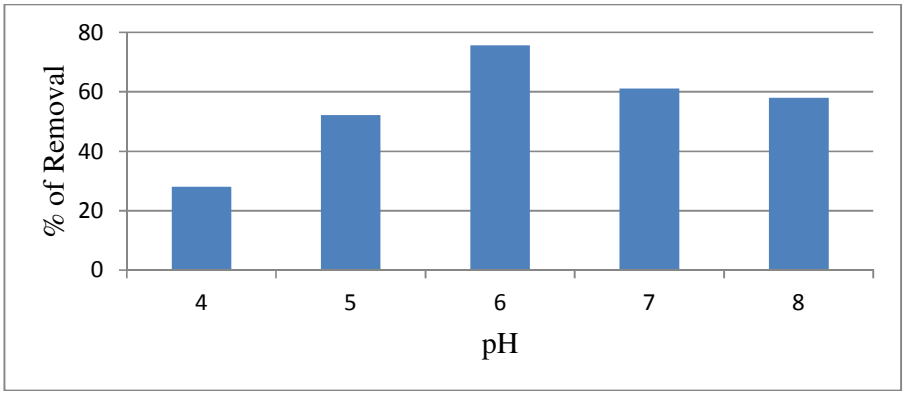

Figure 2: Percentage of Removal of Pb against pH by *Aspergillus terreus*

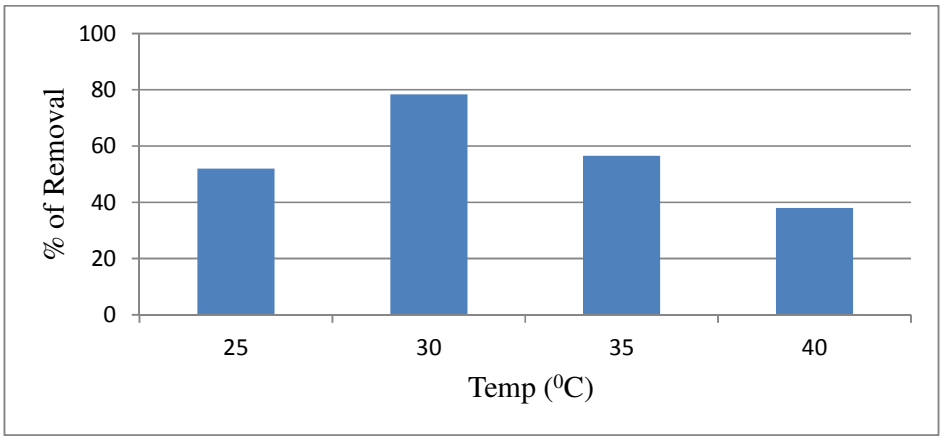

Figure 3: Percentage of Removal of Pb against Temperature

For the effect of inoculums size of *Aspergillus terreus*, different inoculum size of biomass were inoculated, in order to ascertained the bioremoval of Pb. The highest removal was found at 8 mL of the inoculums biomass was found to remove 80.54% of the Pb represented in fig. 4. Kapoor and Viraraghava, (1997) which reported that the increase of mycelia by mass of *Aspergillus niger* lead to the increase in the biosorption activities of the heavy metals due to the contacts of fungal biomass and the metals. In case of incubation time there was increased in the removal of Pb from 72 – 96 h, the highest removal was obtained at 120 h removal of 84.08%, with the dried biomass obtained was 1.78 mg. This corresponds to the Badar *et al*. (2001) who reported biosorption of Zn by *A. niger* and *Penicillium* spp reaching maximum limit was reached within 120 h of incubation with 98.06% of removal. Khan *et al.* (1998) reported that contact time of fungi biomass affect the absorption of the metals in the liquid medium. The fungal isolate shown to entrapped the Pb ion in its cell structure (Plate 2). Garzali *et al*., (2004) reported effective biosorption of heavy metals and hydrocarbons by consortium of fungal isolates in their cell structures, this enable the microbes to carry out the bioremoval activities.

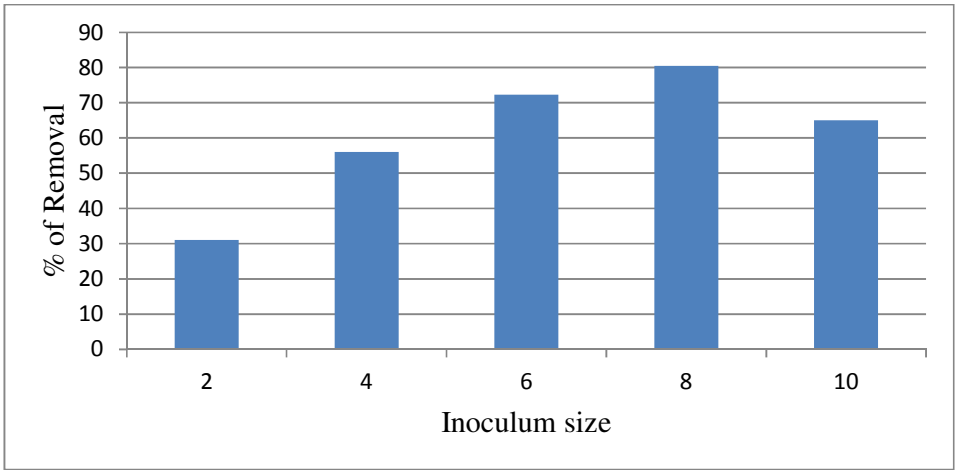

Figure 4: Percentage of Removal of Pb against Inoculum size

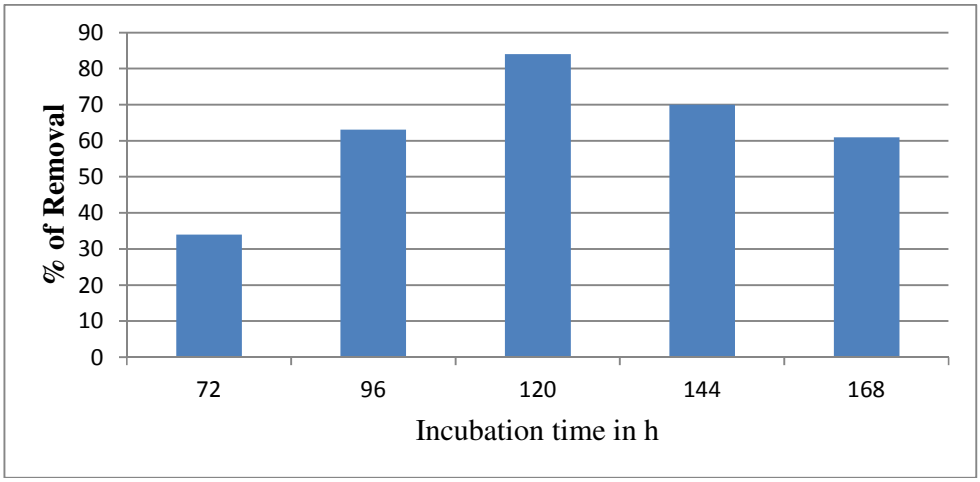

Figure 5: Percentage of Removal of Pb against Incubation time in hour

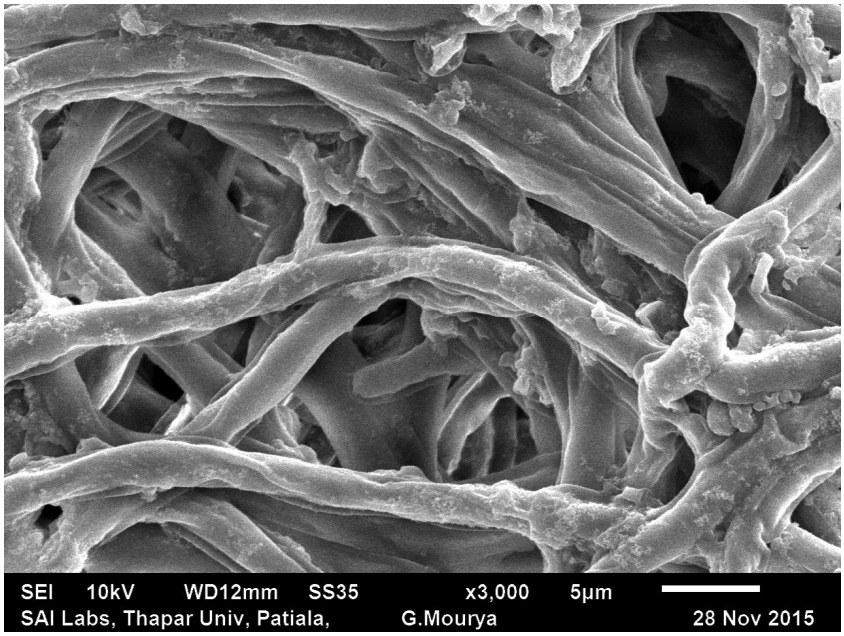

Plate 1**:** Micrograph of *Aspergillus terreus* untreated with Pb

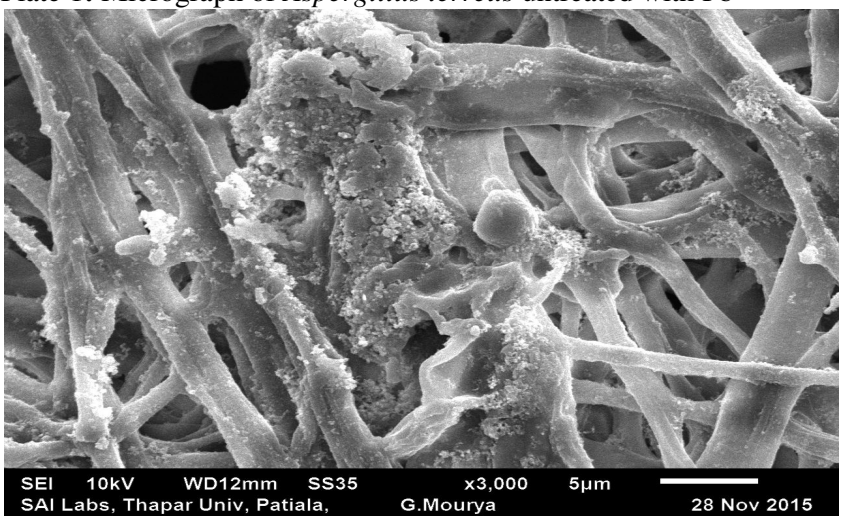

Plate 2: Micrograph of *Aspergillus terreus* treated with Pb

CONCLUSION

The *Aspergillus terreus* was isolated from soil sample obtained from contaminated site at Challawa Industrial Estate, Kano. The isolate was screened using various Pb concentrations and found to be tolerant to the Pb up to 700 mg/L. Optimization of different conditions such as pH, temperature, inoculum size and incubation time were also carried out in order to increase the uptake of the Pb ion. The maximum removal obtained was 84.63%. This indicated that the *Aspergillus terreus* can be potential isolates for reclaiming soils and water that are contaminated by heavy metals from industries. The bioremediation technology is efficient method, which is less costly and environmentally friendly.

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