

Heavy metal tolerance in the psychrotolerant *Cryptococcus* sp. isolated from deep-sea sediments of the Central Indian Basin

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Abstract

Deep-sea isolate of the psychrotolerant yeast *Cryptococcus* sp. (NIOCC #PY13) obtained from polymetallic nodule-bearing sediments of the Central Indian Basin was examined for its capacity to grow in the presence of various concentrations of the heavy metal salts i.e. ZnSO₄, CuSO₄, Pb(CH₃COO)₂ and CdCl₂. It demonstrated considerable growth in the presence of 100 mg l⁻¹ concentration of the above mentioned four heavy metal salts both at 30 as well as 15°C. Optimum pH for growth of this isolate ranged from 6 to 8 in the presence of heavy metal salts at these two temperatures. Scanning electron microscopic (SEM) studies exhibited altered cell surface morphology of the cells under the influence of heavy metals compared to that with control. The adsorption of heavy metals to the cells was demonstrated by FTIR and EDAX analysis. As evidenced by atomic absorption spectrophotometric (AAS) analysis, about 30-90% the heavy metals were removed from the culture supernatant after 4 days of growth at 30°C. This deep-sea yeast isolate appears to be a potential candidate for bioremediation of metal contaminated sites. Moreover, its metal tolerance properties provide a significant insight on its ecological role and adaptations to grow in such extreme conditions.

Key words: Heavy metals, Biosorption, Metal tolerance, Deep-sea sediments, Central Indian Basin, EDAX, FTIR, SEM, *Cryptococcus* sp.

Running title: Heavy metal tolerance in deep-sea yeast

Introduction

Presence of large amount of metals in the sediments of deep-sea hydrothermal vent is a major feature (Malahoff, 1985). However, very little is known about the presence of heavy metals in the non-vent sediments of deep sea. A few studies have reported some of the heavy metals like Cr, Pb, Zn, Co to be present in the deep-sea sediments also (Schnetger et al., 2000). Nath et al. (1989; 2005) reported the presence of various major and minor elements including Pb, Zn, Cu and Co in deep-sea sediments of the Central Indian Basin. Resistance to heavy metals in heterotrophic bacteria from hydrothermal vent environment is reported by several workers (Jeanthon and Prieur, 1990a; 1990b). Kato et al. (1996), Abe and Horikoshi (2001) and Abe et al. (2004) have reported potential biotechnological applications of metal-resistant bacteria from deep-sea sediments. Metal resistant bacteria have been isolated and characterized from sediment of deep-sea Mn nodules of the Pacific Ocean (Tian and Shao, 2006). A Mn(II)-oxidizing deep-sea bacterium was demonstrated to have potential application in multi heavy-metal removing systems (Wang et al., 2009). One of the yeasts belonging to *Cryptococcus sp.*, isolated from deep-sea sediments of the Japan Trench was demonstrated to tolerate very high concentrations of Cu which was triggered by production of an antioxidant enzyme, superoxide dismutase as a defensive mechanism (Abe et al., 2001). The greater capacity of deep-sea isolates towards metal tolerance abilities may be attributed to their ability to survive under extreme conditions by expression of genes involved in combating these stress mechanism (Abe and Minegishi, 2008; Singh et al., 2012). Since the exposure to heavy metals also impose similar stress conditions in microorganisms, deep-sea microbes for the metal tolerance studies may prove highly effective for bioremediation of metal contaminated sites.

Diverse fungal and yeast isolates have been reported from deep-sea sediments of the Central Indian Basin (Damare et al., 2006; Singh et al., 2010). These fungal and yeast isolates exhibited growth under low temperature and elevated hydrostatic pressure conditions (Singh et al., 2010). One of the yeasts NIOCC#PY13 identified as *Cryptococcus sp.* by amplification and sequencing of 18S SSU-rDNA region was psychrotolerant, exhibiting growth at 15°C (Singh et al., 2010). Present study is the first report demonstrating greater tolerance and biosorption characteristics of this deep-sea *Cryptococcus sp.* towards different concentrations of four heavy metal salts. This study opens new avenues to explore the deep-sea organisms for bioremediation capabilities and also provides information regarding their ecological role under such extreme conditions.

Materials and Methods

Yeast strain and culture conditions

The psychrotolerant yeast NIOCC#PY13 (*Cryptococcus vishniacii*) was isolated from deep-sea sediment of the Central Indian Basin (10-16.5°S and 72-77°E) on board the Russian research vessel Akademik Boris Petrov during Cruise # ABP26 in December 2006. The isolate was maintained at 5°C on malt extract agar medium by repeated subculturing at an interval of 20-25 days. It was identified by amplification and sequence analysis of partial 18S region of SSU-rDNA. The 18S sequence was submitted to GenBank NCBI database under the accession number EU723510. The culture showed growth at 15 as well as 30°C (Singh et al., 2010).

Effect of different heavy metals on growth pattern of yeast

The yeast isolate was grown in 20 ml YPD medium (0.5% Yeast extract, 1% Peptone and 1% Dextrose) and incubated overnight at 30°C on a rotary shaker incubator at 170 rpm for preparation of inoculum. One ml of this culture was further inoculated into 100 ml of the YPD medium containing 0 (control), 10, 50 and 100 mg l⁻¹ concentration of four heavy metals, ZnSO₄, CuSO₄, Pb(CH₃COO)₂ and CdCl₂. The flasks containing media were incubated at 30°C and 15°C on a rotary shaker at 170 rpm. The growth was monitored at intervals of 24 h by taking the absorbance at 600 nm. Controls were incubated with four concentrations of the above metal salts in the YPD media without any culture inoculum to monitor the precipitation of these salts and the readings were subtracted from respective media flasks containing the yeast culture. All the experiments were performed in triplicates and the results are represented with average values.

Minimum inhibitory concentration (MIC) values were determined by streaking the isolated colonies of yeast on YPD agar medium plate containing 10 mg l⁻¹ concentration of four heavy metals, ZnSO₄, CuSO₄, Pb(CH₃COO)₂ and CdCl₂. These media plates were incubated at 30°C for 48 hours. This process was repeated with successively higher concentrations of the above four metal salts until the MIC of each metal was obtained. The MIC is defined as the lowest concentration of the heavy metal, added to a media plate at which the yeast isolate streaked from a single colony did not grow.

Effect of pH on the growth of NIOCC#PY13 in the presence of heavy metals

To analyze the effect of pH on growth of yeast in the presence of the above four heavy metals, the inoculum was prepared in a similar way as described above. A range of pH values were selected (2, 4,

6, 8 and 10) for the experiment. The pH of the growth medium was adjusted to the required values using 0.1N HCl or 0.1N NaOH. One ml of the inoculum was added into 100 ml of the YPD medium with different pH values containing 100 mg l⁻¹ concentration of four heavy metals, ZnSO₄, CuSO₄, Pb(CH₃COO)₂ and CdCl₂. The growth was measured by taking the absorbance at 600 nm after 3 and 7 days of incubation at 30°C and 15°C. Controls for each pH values were incubated to monitor the precipitation of these metal salts as described in the previous section.

Effect of heavy metals on the cell morphology

The yeast isolate was grown in the presence of 100 mg l⁻¹ concentration of the heavy metals at 30°C for a period of 3 days. The cell suspension was fixed on small glass pieces and dehydrated sequentially with a range of ethanol solutions (10, 20, 30, 50, 70 and 90%), prepared with distilled water. The cell morphology was observed with a scanning electron microscope (JEOL SEM, Model 5800L, Japan). The acceleration voltage was constant at 20 kV and the microprobe was focused at a magnification of 190×, 230× and 250×. The samples were coated with a thin layer of gold using sputter coater (SPI-MODULE Gold Sputter Coater) to increase the electron conduction and to improve the quality of the micrographs.

Analysis of heavy metals sorption to yeast cell surface

Energy dispersive X-ray analysis (EDAX), and fourier transform infrared spectroscopy (FTIR) were employed to examine the sorption mechanism of this yeast isolate. It was grown in the presence of 100 mg l⁻¹ concentration of the heavy metals at 30°C for a period of 3 days. The cell suspension was centrifuged at 8000 rpm for 10 min at 4°C. The cell pellet was lyophilized for 24 h and the dried biomass was subjected to EDAX and FTIR analysis. EDAX was performed in a similar manner like SEM as described in the previous section by scanning electron microscope (JEOL SEM, Model 5800L, Japan).

Fourier transform infrared spectroscopy (FTIR) spectra were recorded between 4,000 and 500 cm⁻¹ using FTIR (model 8201PC, Shimadzu, Japan) with 1 cm⁻¹ resolution. Pellets were prepared by mixing the lyophilized samples with 50 mg KBr using diffused reflectance spectroscopy accessory. FTIR spectra of lyophilized biomass before and after sorption were recorded.

Analysis of residual heavy metal ions in the culture supernatant

The yeast isolate was grown in the presence of 3 different concentrations of the heavy metal salts (10, 100 and 200 mg l⁻¹) for 3 days. The concentration of residual heavy metal ions (Cu, Pb and Zn) in the supernatant was determined by using a Flame Atomic Absorption Spectrophotometer (Thermo Electron Corporation, S-Series, SOLAR S AAS) with an air–acetylene flame at specific wavelengths for all of the above elements. Blanks and standards were run in a similar manner for calibration. Deuterium background correction was used. The instrument was calibrated by running blank and standard solutions prior to each element analysis. Recalibration check was performed at regular intervals after every 10 readings. All chemicals used in the study were of analytical grade. As the standards with comparable matrix were not available, certified reference standard from the United States Geological Survey (Nod A-1: Nodule standard) was digested and run to test the analytical and instrument accuracy during the time of analyses. The concentration of residual Cd metal ions could not be estimated due to non availability of Cd lamp for the above Spectrophotometer.

Results and Discussion

Effect of temperature and pH on metal tolerance properties of the yeast

The yeast isolate used in the present study exhibited considerable growth in the presence of all the three concentrations (10, 50 and 100 mg l⁻¹) of the four heavy metals, Pb, Cu, Zn and Cd. The growth with 100 mg l⁻¹ of heavy metals was comparable to the control, both at 30 as well as 15°C (Fig. 1a and 1b). However, at 15°C, the time taken to reach the stationary phase was longer than at 30°C. The order of toxicity was found to be Cd>Cu>Zn>Pb for the growth at these two temperatures. A similar observation has been reported where yeast strains were found to tolerate higher concentration of Zn than Cu (Vadkertiova and Slavikova, 2006). However, the level of Cu tolerated by the deep-sea yeast in the present study was comparatively higher which is in concordance with the results obtained by Abe et al. (2001) where a deep-sea *Cryptococcus* sp. could grow on the solid medium containing 50 mM CuSO₄. The minimum inhibitory concentration of the four heavy metals used in the present study, ranged from 900-1400 mg l⁻¹. These results demonstrated high level of heavy metal tolerance of this deep-sea yeast isolate both at 30 as well as at 15°C.

Optimum pH for the growth of the yeast isolate was found to vary for different metals and temperatures (Fig. 1c). The isolate showed good growth with 100 mg l⁻¹ concentration of Zn, Pb and Cu at pH ranges of 6-8 whereas for Cd, pH 8 was found to be the most favorable (Fig. 1c). A combination of low temperature (15°C) and pH 8 enhanced the growth of the yeast in the presence of

Cd, almost reaching the value of the control. In general, optimum pH for metal uptake has been reported between 4 and 8 for a wide range of microbes (Fourest and Roux, 1992; Brady and Duncan, 1994). Previous studies have reported pH to be a significant factor for the biosorption of heavy metals to microbial biomass, influencing solution chemistry of the heavy metals (Özer and Özer, 2003; Tewari et al., 2005). With increasing pH, relatively more ligands would be exposed carrying negative charges resulting in the subsequent attraction of metal cations and biosorption onto the binding sites on the cell surface (Brady and Duncan, 1994; Delgado et al., 1998). The biosorption capacity of *Mucor rouxii* cells for Pb(II) was found to increase with increasing pH value and reached a maximum at 5.0 (Yan and Viraraghavan, 2003).

Similarly, the yeast isolate used in the present study showed better growth in the presence of different metals with increasing pH with best results at pH 6.0 for Pb, Zn and Cu both at 30 as well as 15°C (Fig. 1c). This suggests that at this pH the cell surface ligands of the yeast *Cryptococcus* sp. responsible for binding of these three metal cations in the solution are most efficiently exposed. In contrast, pH 8.0 at both the temperatures was found to be suitable for better growth of this yeast in the presence of Cd (Fig. 1c). The composition of extracellular glycoproteins has been reported to influence the adsorption and tolerance level of Cd by different yeast species (Breierová et al., 2002). A similar phenomenon may be suggested for enhanced tolerance towards Cd at low temperature and pH 8.0 in the present study by alteration of extracellular glycoproteins responsible for Cd uptake on yeast cell surface.

Effect of heavy metals on cell morphology

SEM micrographs of the yeast indicated clear changes in cell surface morphology after growth in the presence of the heavy metals (Fig. 2). The clear damage to yeast (Fig. 2a) could be observed by the presence of shrunken and distorted cell wall in the presence of Cd (Fig. 2b) and depressions in the presence of Pb and Zn (Figs. 2d and 2e). Least visible damage was noticed with Cu loaded yeast cells (Fig. 2c). Although the cell morphology was affected in the presence of the heavy metals their overall growth was not much influenced. Various factors may be responsible for such alterations in cell surface morphology of microbial biomass in the presence of heavy metals. Secretion of extra cellular polymeric substance by *Desulfovibrio desulfuricans* during biosorption of Zn and Cu was reported to modify its cell surface morphology (Chen et al., 2000). Biosorption of Zn, Ni and Cr was demonstrated by SEM analysis using fungal biomass of *Aspergillus* sp. (Kumar et al., 2012).

EDAX analysis for biosorption of heavy metals

EDAX spectrum of the deep-sea yeast grown in the presence of three different heavy metals (Cd, Cu and Pb) showed the presence of corresponding metal peaks (Fig. 3) demonstrating their biosorption on cell surface. No positive result was observed for EDAX spectra of the yeast cells grown in the presence of Zn. One of the possible explanations for this may be the very different texture of yeast biomass after growth in the presence of Zn. The yeast cells turned in to powdered form when dried after growth with Zn in the media and did not stick to the sputter causing undetected peaks in EDAX spectra. EDAX spectra have been implied successfully for the manifestation of metal biosorption by microbial biomass in the earlier studies (Raize et al., 2004; Çabuk et al., 2007). However, a much more detailed study involving metabolic phases of the yeast is recommended in future which could provide a clear picture on metal uptake mechanism.

FTIR spectrum analysis

FTIR spectra of the pure biomass of yeast isolate was compared with the biomass loaded with 100 mg l⁻¹ concentration of the four heavy metals to examine the functional groups changes due to metal ions interaction (Fig. 4). Several new peaks appeared in the spectral pattern of metal- loaded biomass compared with the control. Spectral changes in Pb and Zn- loaded biomass were prominent in the region from 800 to 1700 cm⁻¹. However, Cd and Cu- loaded biomass exhibited comparatively lesser number of peak shifts than control biomass (Figs. 4a, b and c). The spectral changes in the region, 1600-1700 cm⁻¹ were observed in all the 4 heavy metal- loaded biomass of the yeast isolate (Fig. 4a-4e). The peak range from 1500 to 1700 cm⁻¹ is stretching vibration band of C=O of amide bond suggesting stretching of this functional group as a result of metal biosorption. Similar results were reported by Çabuk et al. (2007), where amide groups were found to be involved in biosorption of Pb(II) ions by indicating a peak shift at 1633 cm⁻¹ in the FTIR spectrum of Pb- loaded *S. cerevisiae* biomass.

The new peaks appeared in the 1500–1200 cm⁻¹ for all the metals in the present study representing C-H bending vibrations of CH₃, CH₂ and CH functional groups (Wolkers et al., 2004). The peak shifts in spectral region from 1200 to 900 cm⁻¹ corresponds to C-O, C-C, C-O-C and C-O-P stretching vibrations of polysaccharides. The change of peaks in this region in the presence of all the four metals suggests interaction of cell surface polysaccharides to metal ions facilitating their biosorption. A stretching peak of 2367 cm⁻¹ is assigned to the asymmetric stretching of the isocyanate

group (-N=C=O) (Gholap and Badiger, 2004). The yeast isolate demonstrated stretching of peak at 2362 cm^{-1} , indicating the involvement of N=C=O group in the biosorption of Pb (Fig. 4d). Broad spectral bands in the region of $3700\text{-}3300\text{ cm}^{-1}$ demonstrate N-H or O-H stretching vibrations (Guo and Zhang, 2004) and alkyl chains are represented by other bands around $2920\text{-}2850\text{ cm}^{-1}$. A peak shift in the region 3300 cm^{-1} was observed for all the metal treated biomass suggesting N-H or O-H stretching vibrations. Also, peak shift around 2800 cm^{-1} represented -CH stretching vibration of -CH₃ and -CH₂ groups during biosorption of these heavy metals.

Metal removal properties of the yeast isolate

Considerable percentage of heavy metals was removed from the culture supernatant by the yeast isolate. However, the metal uptake capacity varied with the initial concentration of the metal added to the solution, the highest being 98% removal in the presence of 10 mg l^{-1} of Pb and Zn (Fig. 5). Minimum removal was observed for the solution containing 200 mg l^{-1} Cu and was found to be 30% (Fig. 5). The yeast, *Lodderomyces elongisporus* was shown to remove 81% of Cu from the medium, added at a concentration of 0.1 mg l^{-1} after 96 h of incubation (Rehman et al., 2008). Several other studies have also emphasized the role of bacteria (Shakoori and Muneer, 2002), fungi (Dönmez and Aksu, 2001) and algae (Feng and Aldrich, 2004) towards tolerance or bioaccumulation of Cu from metal solutions. Among fungi, most of the yeast isolates have been found to be sensitive to higher concentration of Cu except a deep-sea *Cryptococcus* sp., which could tolerate 50 mM concentration of CuSO₄ (Abe et al., 2001). The results of the present study are also in accordance with the above observation where a deep-sea yeast tolerated $\sim 10\text{ mM}$ concentration of CuSO₄ in solution and successfully removed $\sim 80\%$ of Cu from a 10 mg l^{-1} ($\sim 0.1\text{ mM}$) solution (Fig. 5). However, Abe et al. (2001) demonstrated the growth of yeast on media plate with 50 mM concentration of CuSO₄, whereas in the present study metal tolerance of yeast was shown in liquid media. The yeast may exhibit different growth patterns in the presence of metals on solid and liquid media.

The results of the present study suggest greater capability of this deep-sea isolate for different heavy metal removal from solutions which may find application in bioremediation of metal contaminated sites. Further studies involving metabolic phases of this yeast are suggested for future research after exposure to heavy metals which will better elucidate the understanding of biosorption mechanism. Up- and downregulated genes under simulated deep-sea conditions were studied in this isolate (Singh et al., 2012). It will be of interest to look for such genes in the presence of heavy metal

stress and any commonality of such genes under combination of these various stress conditions.

Conclusion

In conclusion, the deep-sea *Cryptococcus* sp., exhibited remarkable tolerance and biosorption towards different concentrations of the four heavy metals tested. The optimum temperature and pH range for its growth in the presence of metals was found to be 30°C and 6-8 respectively. Altered cell surface morphology in the presence of heavy metals was observed by SEM. The uptake of metals and the bonds involved in metal ion interaction onto the cell surface were demonstrated by EDAX and FTIR analysis respectively. In addition, a high percentage of metals were removed from the culture medium by this yeast isolate. All these results suggest that this deep-sea yeast contains significant metal tolerance and biosorption capabilities and may be used for bioremediation of metal contaminated sites. Being isolated from an environment rich in polymetallic nodules, this isolate may have evolved a defensive mechanism to detoxify the environment by bioaccumulation of heavy metals and develop tolerance to them. A detailed study on metabolic approaches adopted by this yeast during metal uptake process may provide greater insight on its ecological role in such deep-sea sediments.

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Figure legends:

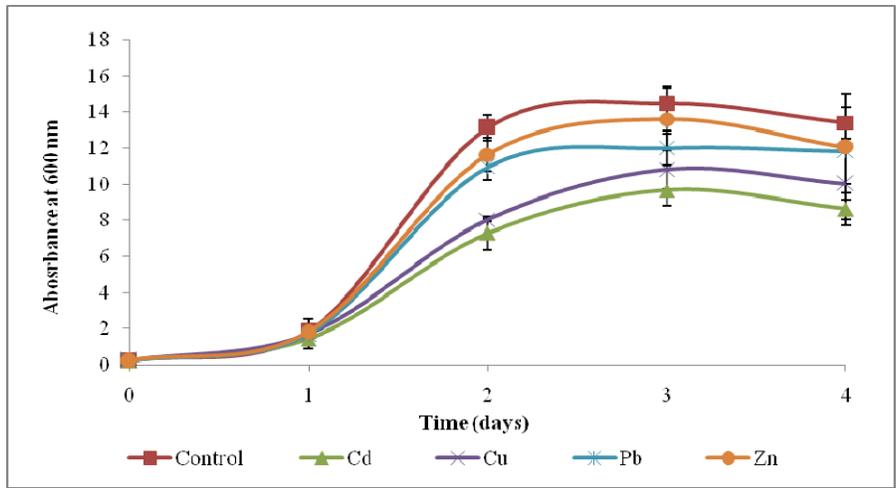
Fig. 1. Growth pattern of the deep-sea yeast isolate, NIOCC#PY13 in the presence of 100 mg l⁻¹ concentration of four heavy metals at: a) 30°C; b) 15°C and c) different pH and temperature conditions.

Fig. 2. Scanning electron microscopic pictures of NIOCC#PY13 grown in the presence of different heavy metals at a concentration of 100 mg l⁻¹.

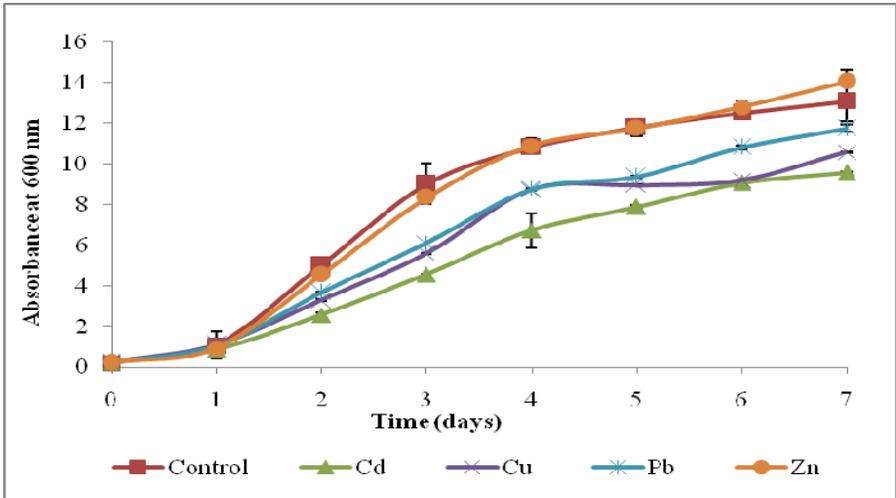
Fig. 3. EDAX spectra of NIOCC#PY13 representing biosorption of different heavy metals: a) control; b) Cd; c) Pb and d) Cu. All of these heavy metals were used at a final concentration of 100 mg l⁻¹. EDAX spectra of NIOCC#PY13 in the presence of Zn did not show any significant peaks and therefore has not been represented in the figure.

Fig. 4. The FT-IR spectrum patterns of deep-sea *Cryptococcus* sp.; control (a) and metal loaded biomass; b) Pb; c) Zn; d) Cd and e) Cu.

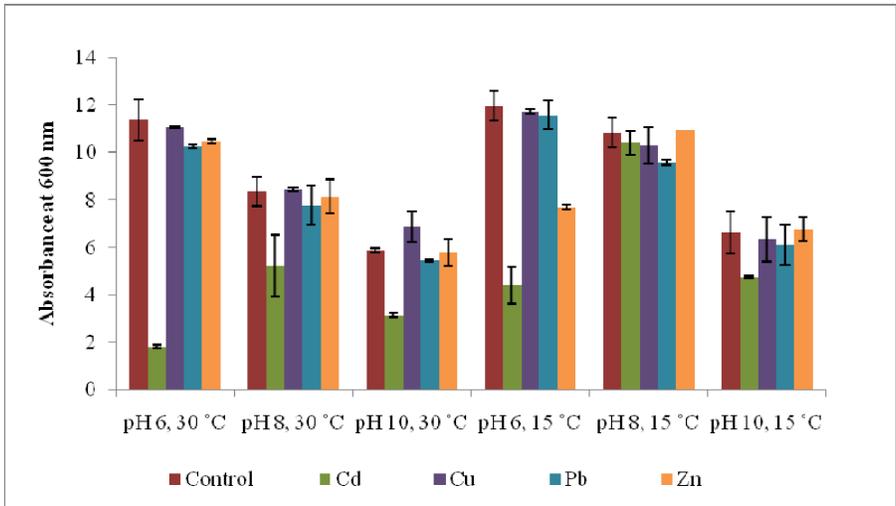
Fig. 5. Percentage removal of metals from culture filtrate by NIOCC#PY13 for three different concentrations of heavy metals. The results for percentage removal of Cd has not been included as the analysis could not be done due to non-availability of Cd lamp in the spectrophotometer.



a)

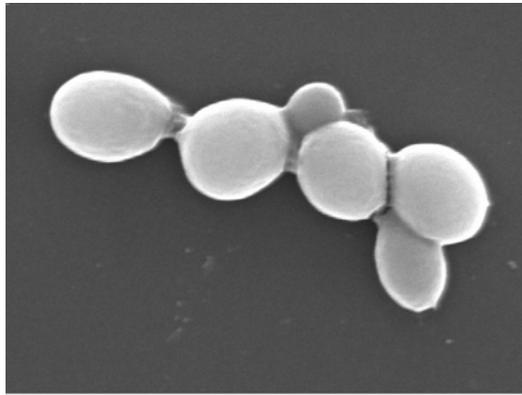


b)



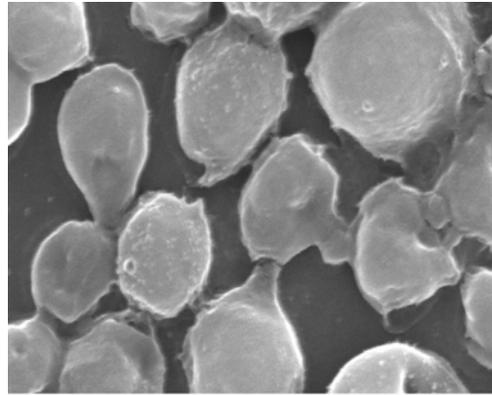
c)

Fig. 1



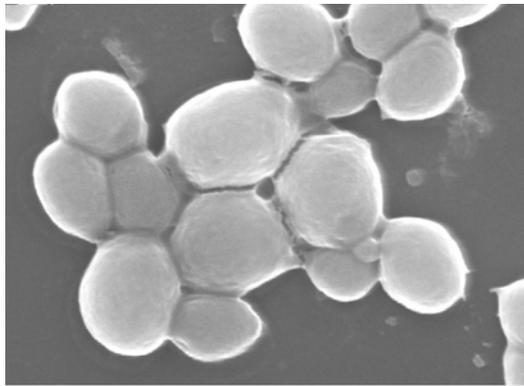
6μm

a) Control



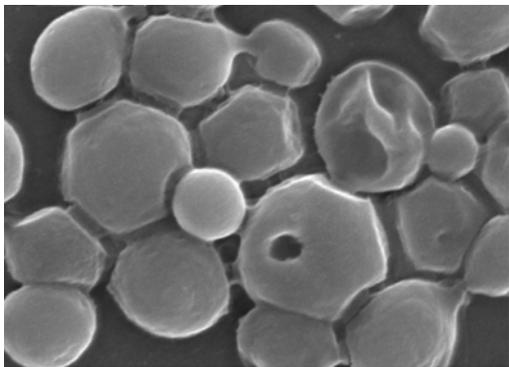
6μm

b) Cd



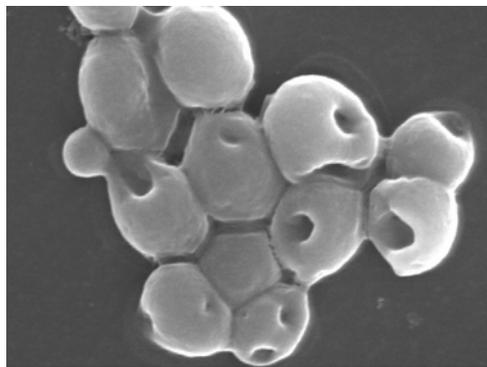
6μm

c) Cu



6μm

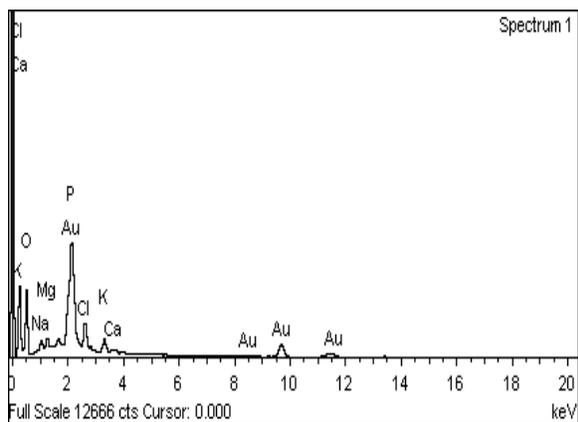
d) Pb



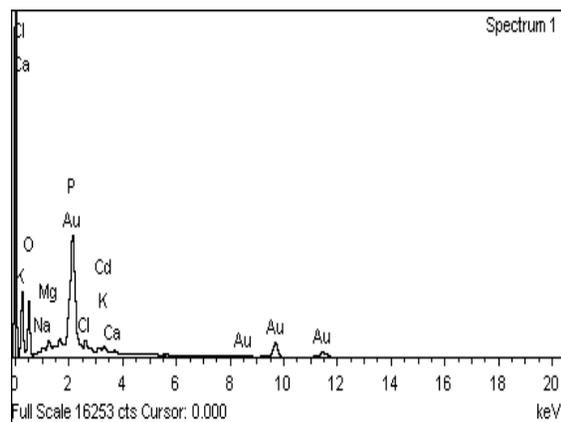
6μm

e) Zn

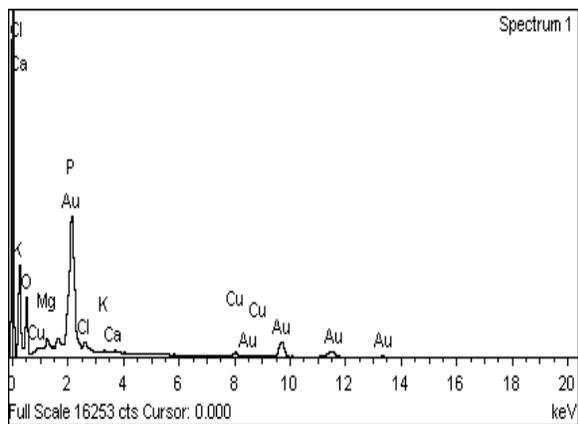
Fig. 2



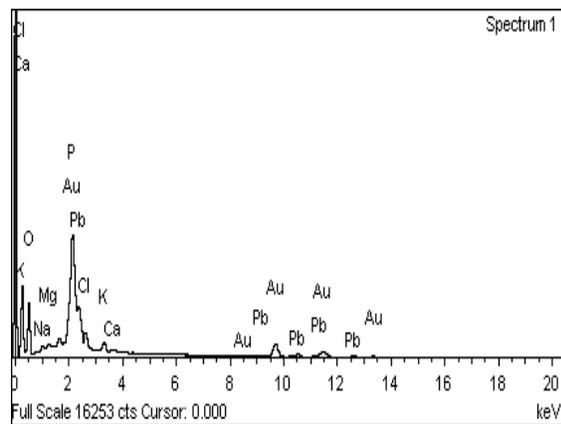
a) Control



b) Cd



c) Cu



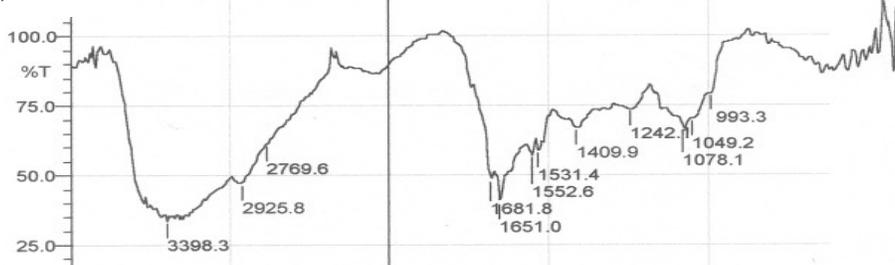
d) Pb

Fig. 3

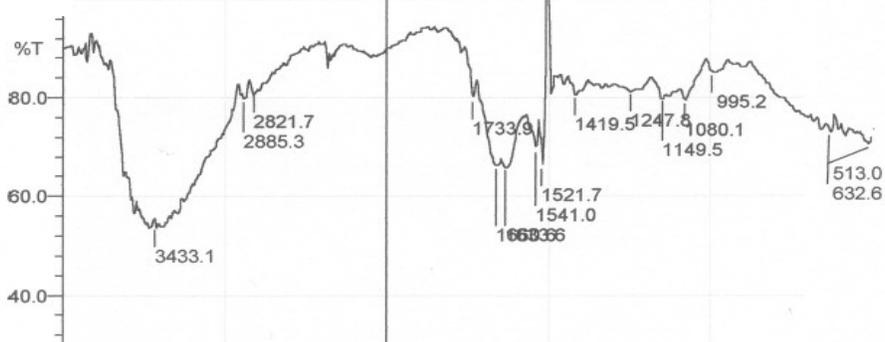
a) Control (pure biomass)



b) Cd



c) Cu



d) Pb



e) Zn

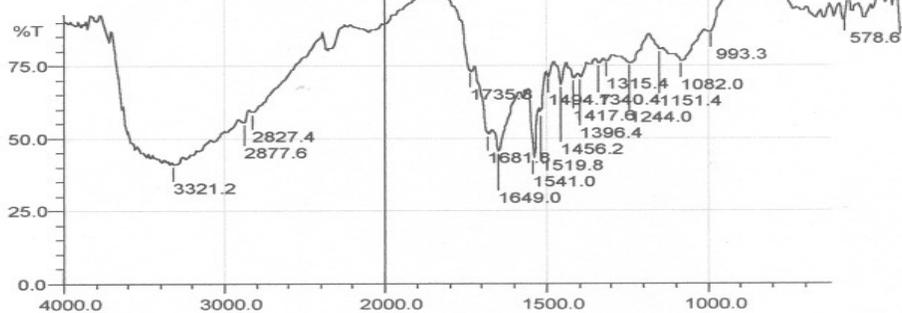


Fig. 4

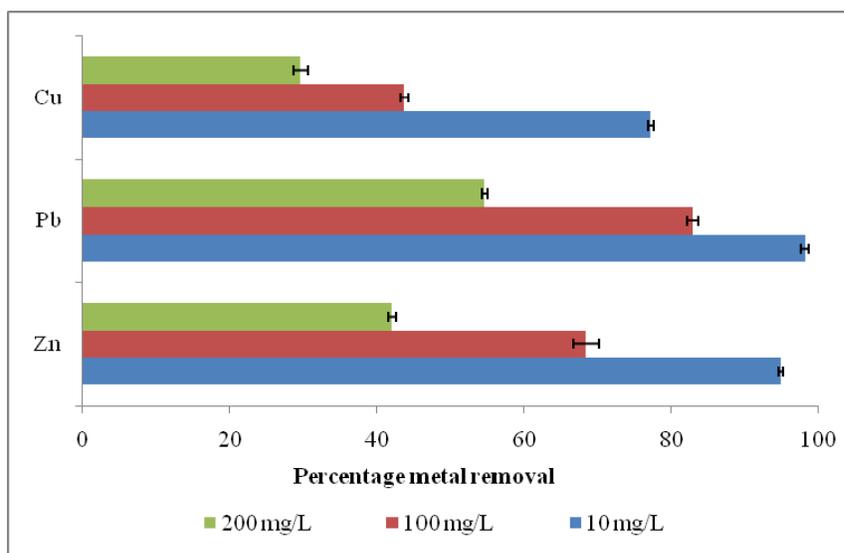


Fig. 5