African Journal of Biotechnology Vol. 11(42), pp. 9993-10001, 24 May, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.4282
ISSN 1684–5315 © 2012 Academic Journals

*Full Length Research Paper*

# Biogenic volatile compounds of activated sludge and their application for metal bioremediation

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Accepted 20 April, 2012

**Heavy metals pollution is nowadays one of the most important environmental concerns. This paper illustrates the employment of the biogenic volatile compounds generated during the aerobic growth of activated sludge on raw domestic wastewater for heavy metals removal. Most of the tested metals even as individual or mixed metal species (Co, Cu, Cd, Fe, Hg, Ni, Mn, Pb and Zn) were potentially transformed into insoluble precipitates and then separated out of their solutions. The Fourier-transform infrared (FTIR) analysis has identified some organosulfur groups (thiol, disulfide and thiocarbonyl), in addition to amine group in the metal precipitates. This study highlighted the application of the microbial volatile metabolites for heavy metals bioremediation in a powerful, cost effective and eco-friendly bioprocess.**

**Key words:** Application, activated sludge, biogas, metals, bio-precipitation.

## INTRODUCTION

Anthropogenic activities like mining, processes of metallurgy and other chemical industries lead to the discharge of a variety of persistent metals into the environment that causes serious problems to human health. In developing countries, careless fulfillment of laws and lack of an economic method for industrial waste management have caused rising pollution of water sources.

The biological methods of heavy metal removal have proven to be supreme as compared to the physical and chemical processes (Ilhan et al., 2004; You et al., 2010). Microorganisms play a crucial role in bioremediation of heavy metals from contaminated soil and wastewater since they are easy to operate, do not produce secondary pollution and show higher efficiency at low metal concentrations (Chen et al., 2005; De et al., 2008). Although, microorganisms cannot destroy metals, they can alter their chemical properties through several mechanisms such as biosorption, bioleaching, biomineralization, intracellular accumulation and enzyme-catalyzed transformation (Lloyd, 2002; Gadd, 2010).

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Since most of the microbial cells are sensitive against the aggressive industrial wastewater due to high levels of toxic metals, extreme pH values, and presence of other pollutants that may kill or suppress the microbial growth, the direct metal-microbe interaction become less effective for metal bioremediation under these extreme conditions. Consequently, a potential commercial importance rely on the indirect metal-microbe interaction that assists microorganisms to tolerate high concentrations of heavy metals in their environment through producing a wide variety of compounds that can participate in the precipitation of these metals away from the cells (Gadd, 2010).

The microbial liberation of extracellular non specific compounds such as oxalates, sulfides, phosphates and carbonates, in addition to some organic substances that are produced during the microbial cell metabolism, play an important role in the reduction of heavy metals toxicity through the bioprecipitation of these pollutants in the microbial environment (Jarosz-Wilkolazka and Gadd, 2003; Perry et al., 2007; Fomina et al., 2008; Dupraz et al., 2009).

A group of these extracellular compounds are formed during the primary and/or secondary microbial metabolism in the form of volatile etabolites such as

alcohols, ketones, terpenes, esters, aldehydes, mercaptans and nitrogen-based compounds (Hockelmann and Juttner, 2004; Bunge et al., 2008). Production of these compounds is greatly affected by microbial species and growth conditions (Wood et al., 2006; Schafer et al., 2010). Although the function of the microbial volatile compounds still not clear, some studies showed that they could play a role in the precipitation of some heavy metals as insoluble metal complexes (Macaskie et al., 2007).

So the objective of this study was to establish an economic bioprocess for heavy metals elimination via using the volatile metabolites of the bacterial biogas.

## MATERIALS AND METHODS

### Isolation and purification of bacterial strains

One milliter of activated sludge sample (Sewage Disposal Station, Beyahmo, Fayoum, Egypt) was dissolved in 99 ml sterile distilled water and serial dilutions were made. Each dilution was plated on nutrient agar plate without antibiotics and incubated at 30°C for 48 h. After the growth of the different microorganisms, each bacterial colony on the basis of its morphological characteristics was picked up and further purified by repeated streaking on nutrient agar plates. The isolated bacterial strains were kept at -80°C in the presence of sterile glycerol.

### Morphological, physiological and biochemical characterization

Cell morphology was determined by phase contrast microscopy. Catalase, oxidase and the starch hydrolase activities were determined as described by Cowan & Steel (1965). The hydrolysis of gelatin and urea were studied as well. API ZYM and API 50CHB systems (API bioMerieux) were used to determine phenotypic and enzymatic activities. For rapid detection of methanethiol production, the method of Pitcher and Malnick (1984) was used and slants were inspected after 24 h. Diffusion of a bright yellowness through the medium was taken as a positive result. The ability of the bacterial isolates to produce ammonia was confirmed by using Nessler's solution which gives a distinct yellow coloration in the presence of the least trace of ammonia.

The metal tolerance profile of the isolated bacteria was carried out according to (Choundhury and Kumar, 1996). Overnight grown cultures of the bacterial strains were inoculated with the following metals [$Ni_2SO_4$ (0.2 mg/mL), $CoCl_2$ (0.5 mg/mL), $CdCl_2$ (0.2 mg/mL), $CuSO_4$ (0.5 mg/ml), $HgCl_2$ (10 µg/ml), $Pb(NO_3)_2$ (1.2 mg/ml), $ZnSO_4$ (0.5 mg/ml), $FeCl_3$ (0.5 mg/ml), $MnCl_2$ (0.5 mg/ml)] and incubated at 30°C under shaking conditions (150 rpm) for 16 h. The bacterial growth was recorded as optical density at 600 nm ($OD_{600}$) on a Phamacia LKB, Novaspac II spectrophotometer.

### Metal solution preparation

Stock solutions of the tested metals were prepared separately by dissolving 500 mg of the following salts $Ni_2SO_4$, $CoCl_2$, $CdCl_2$, $CuSO_4$, $HgCl_2$, $Pb(NO_3)_2$, $ZnSO_4$, $FeCl_3$ and $MnCl_2$ in 100 mL of distilled water and then filtered at 0.2 µm. From these solutions, the different metal concentrations were prepared either separately or in combination.

### Operation of the bioreactor

For metal bioprecipitation experiments, a bioreactor (Figure 1) was used according to Essa et al. (2005). The bioreactor composed of two chambers. The growth chamber (1.5 L) that contains the bacterial culture grew on Luria Bertani (LB) medium or sterilized raw domestic wastewater obtained from Sewage Disposal Station, Beyahmo, Fayoum, Egypt (Table 1). The growth chamber was inoculated with 150 mL of the different bacterial stains in the exponential phase (6 h) or the fresh activated sludge, and maintained under aerobic conditions at 30°C by pumping in filtered compressed air. The other chamber was used for metal precipitation (250 ml) by passing the culture biogas released from the growth chamber into the metal solution as a single or mixed metal solution with different concentrations (Figures 1, 2 and Table 3) for 24 h through a 0.2 µm membrane. The cell density of the bacterial liquid cultures was determined as mentioned above.

### Heavy metals determination

After treating the metal solutions with the biogenic volatiles produced by bacterial cultures for 24 h, the metal solutions collected from the precipitation chamber were centrifuged at 10,000 rpm, for 5 min, and the supernatant was used for the determination of the different heavy metals, while the metal precipitate pellets were subjected for Fourier-transform infrared (FTIR) spectroscopy. The mercury ion concentration was assayed by using Perkinelmer 3300 Spectrometer Atomic Absorption (using hydride system) (Water, Soil and Environment Research Institute, Agriculture Centers, Ministry of Agriculture), while the other ions $Ni^{2+}$, $Co^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $Fe^{3+}$, $Cd^{2+}$, $Pb^{2+}$ and $Cu^{2+}$ were determined using Unicam Solar 989 AA Spectrometer Atomic Absorption (Faculty of Agriculture, Cairo University).

### FTIR spectroscopy

The metal precipitate pellet produced from the previous step was washed three times in distilled $H_2O$ and then dried in vacuum oven to obtain metal precipitate in powder form that was analyzed by FTIR spectrophotometer at the wave number range of 400.00 $cm^{-1}$ to 4000 $cm^{-1}$ (Faculty of Science, Fayoum University, Egypt).

## RESULTS AND DISCUSSION

### Isolation, identification and characterization of bacteria

The bacterial strains (B1, B2, B3, B4 and B5), isolated from active sludge samples obtained from Sewage Disposal Station of Beyahmo, Fayoum, Egypt, were identified on the basis of their morphological and biochemical characteristics (Buchanan and Gibbons, 1974) (Table 2) as *Micrococcus halobius, Streptococcus mutans, Aureobacterium barkeri*, *Bacillus cereus and Bacillus globisporus*, respectively. At the same time, these isolates were screened for tolerance against some bio-essential micronutrients as zinc, manganese, iron, cobalt, nickel and copper that might be toxic at high concentrations, as well as some highly toxic metals as mercury, cadmium and lead. The bacterial isolates demonstrated variable degrees of tolerance to the tested

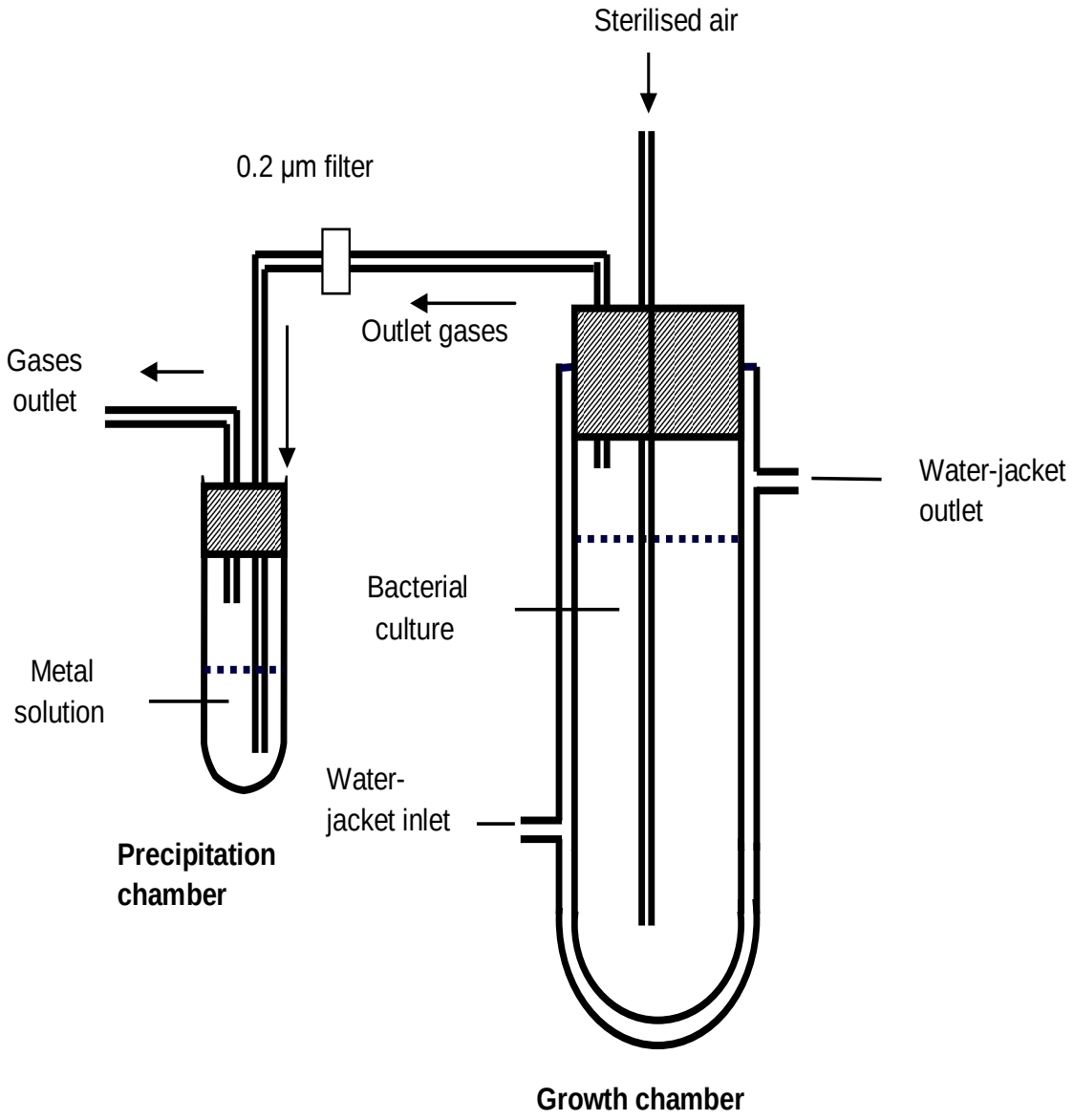


**Figure 1.** A bioreactor used for metal precipitation through the culture off-gas consisting of two chambers. The large vessel is the bacterial growth chamber and the second is the metal precipitation chamber. The growth vessel was held at 30°C through the water jacket and an external water bath.

**Table 1.** Raw domestic wastewater analysis.

| Parameter | Value |
|---|---|
| pH | 6.5 |
| BOD* ($mgL^{-1}$) | 184.9 |
| COD** ($mgL^{-1}$) | 229.5 |
| TSS** ($mgL^{-1}$) | 72 |
| Nitrate ($mgL^{-1}$) | 5.7 |
| Ammonia ($mgL^{-1}$) | 31.4 |
| Phosphate ($mgL^{-1}$) | 3.2 |

***,** Biological oxygen demand; **, chemical oxygen demand; ***, total suspended substances.

**Table 2**. Morphological and biochemical characteristics of the bacterial strains (B1, B2, B3, B4 and B5) that were isolated from the activated sludge.

| Test | B1 | B2 | B3 | B4 | B5 |
|---|---|---|---|---|---|
| Gram stain | + | + | + | + | + |
| Cell shape | Coccus | Coccus | Rod | Rod | Rod |
| Endospore | - | - | - | Rounded | Ovoid |
| KOH test (Cowan and Steel, 1965) | - | - | - | - | - |
| Oxidase (Cowan and Steel, 1965) | + | - | - | + | + |
| Catalase (Cowan and Steel, 1965) | - | - | + | + | + |
| Amylase (Cowan and Steel, 1965) | + | - | - | - | - |
| Urease (Lanyi, 1987) | - | - | - | - | - |
| Gelatinase (Lanyi, 1987) | - | - | - | - | + |
| Methanthiol (Pitcher and Malnick, 1984) | + | - | + | + | - |
| Ammonia | + | + | + | + | + |
| **Metal tolerance profile*** | | | | | |
| **Metal** | **B1** | **B2** | **B3** | **B4** | **B5** |
| $Pb^{2+}$ | + | - | + | - | - |
| $Zn^{2+}$ | - | + | + | + | + |
| $Fe^{3+}$ | + | - | - | - | - |
| $Mn^{2+}$ | + | + | + | - | + |
| $Co^{2+}$ | + | - | + | + | + |
| $Ni^{2+}$ | - | + | - | - | - |
| $Cd^{2+}$ | - | + | + | - | - |
| $Cu^{2+}$ | + | - | - | - | - |
| $Hg^{2+}$ | - | - | - | + | + |

+, Positive results; -, negative results.

metals (Table 2).

## Comparison between the different isolates for cadmium precipitation

The ability of the isolated bacterial strains for metal precipitation was studied using the biogas produced during their growth in a bioreactor as monospecies cultures, compared with the bacterial population of the activated sludge (Figure 2). In this experiment, cadmium was chosen as a model of heavy metals. Although there were no remarkable changes in the optical density of the different bacterial cultures ($O.D_{600}$ was ranged between 0.9 to 1.1), variable capabilities of cadmium precipitation were recorded. The activated sludge recorded the highest percentage of cadmium removal (99.3%). In case of the monospecies cultures, *A. barkeri, B. globisporus* and *S. mutans* demonstrated a great attitude for cadmium precipitation (95.0, 94.7 and 91.3%, respectively), while *B. cereus* and *M. halobius* achieved a relatively low potential for Cd removal (66.3 and 42.7%, respectively).

## Biogenic volatiles for the precipitation of monospecies metal solution

Biological chelation of metals is a natural mechanism through which the harmful effect of these metals could be reduced by interaction with the numerous molecules possessing groups, capable of complexation or chelation (Gadd, 2010).

The effect of the biogas produced during the growth of the bacterial population on the precipitation of the tested metals at different concentrations after 24 h was showed in Figure 3.

A great metal bioremoval capability for most of the metal as monospecies metal solutions ($Mn^{2+}$, $Fe^{3+}$, $Co^{2+}$, $Hg^{2+}$, $Pb^{2+}$, $Cd^{2+}$ and $Zn^{2+}$) was achieved with the different metal concentrations after 24 h. On the other hand, with the minor concentrations of nickel(II) and copper(II), a shallow bioprecipitation was recorded (5.9 and 9.5%,.

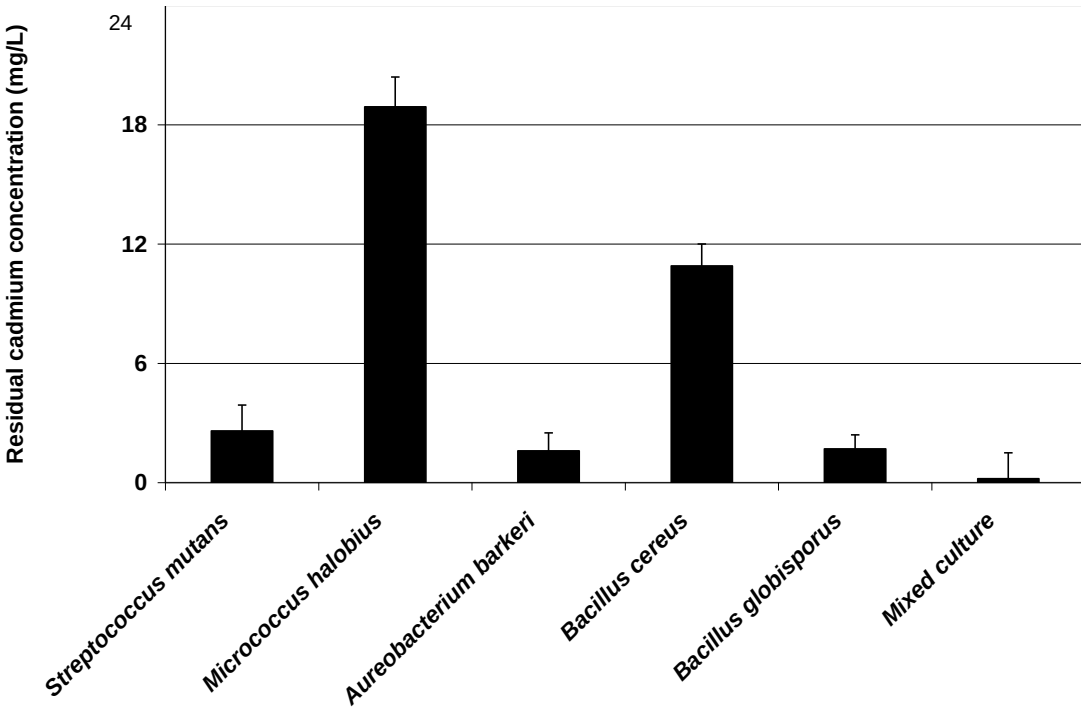


**Figure 2.** A comparison between the different bacterial isolates (*Micrococcus halobius, Streptococcus mutans, Aureobacterium barkeri, Bacillus cereus* and *Bacillus globisporus*) for cadmium bioremoval ($O.D_{600}$ = 1.3±0.8) after 24 h of exposure time. Initial concentration of cadmium was 32.3 mg/L. Data are the means of three replicates and error bars represent the standard errors of the means.

respectively). Moreover, a stumpy rate of copper bioprecipitation was recognised with the different Cu(II) concentrations

## Biogenic volatiles for the precipitation of mixed metal solution

It was assumed that the biogas produced from the bacterial culture contains some volatile metabolites that participate in the metal chelation process. In order to apply this bioprocess for metal cleanup, the bacterial population of the activated sludge was grown aerobically inside a bioreactor and the volatilized biogas was used to precipitate metals out of mixed metal solution (Table 3). Within 24 h, most of the tested metals were potentially precipitated such as iron(III) (98.6%), lead(II) (97.9%), cobalt(II) (95.9%), manganese(II) (94.2%), nickel(II) (94.1%), zinc(II) (85.3%), mercury(II) (81.6%) and cadmium(II) (80.9%), while the lowest rate of precipitation was recorded for copper(II) (60.1%) under the same conditions.

## Analysis of the metal precipitates

The FTIR analyses of the metal precipitate (Figure 4A) have identified some organosulfur groups such as disulfides (SS at 540 $cm^{-1}$) and thiols (SH at 2550 $cm^{-1}$) in the Hg-precipitate. Meanwhile the Cd and Pb-precipitates (Figure 4B) contained disulfides (SS at 540 $cm^{-1}$), thiocarbonyl (CS at 1200 $cm^{-1}$) and amines (NH at 3500 $cm^{-1}$). In case of Cu, Zn, Ni, Co, Fe and Mn-precipitates, the FTIR analysis showed the presence of amine groups only (Figure 4C).

It is clear from the FTIR analysis and data in Table 2 that, the biogas produced during the bacterial population growth contains volatile sulfur and nitrogen-based metabolites. These volatile metabolites might be produced as a result of the catabolism of some amino acids such as methionine (Kadota and Ishida, 1972). Many studies documented a significant production of volatile sulfur compounds as methanethiol, dimethylsulfide and dimethyldisulfide from microorganisms (Kelly et al., 2006; Lefebvre et al., 2007; Boden et al., 2011). The volatile sulfur species of the bacterial biogas have a great tendency to form ligand with some metal ions resulting into insoluble metal complexes. In consistence with that, a previous study (Essa et al., 2006) showed the capability of *Klebsiella pneumoniae* to transform the metal ions of mercury, cadmium and lead into insoluble metal-thiol complexes due to the interaction of dimethyldisulfide identified in the

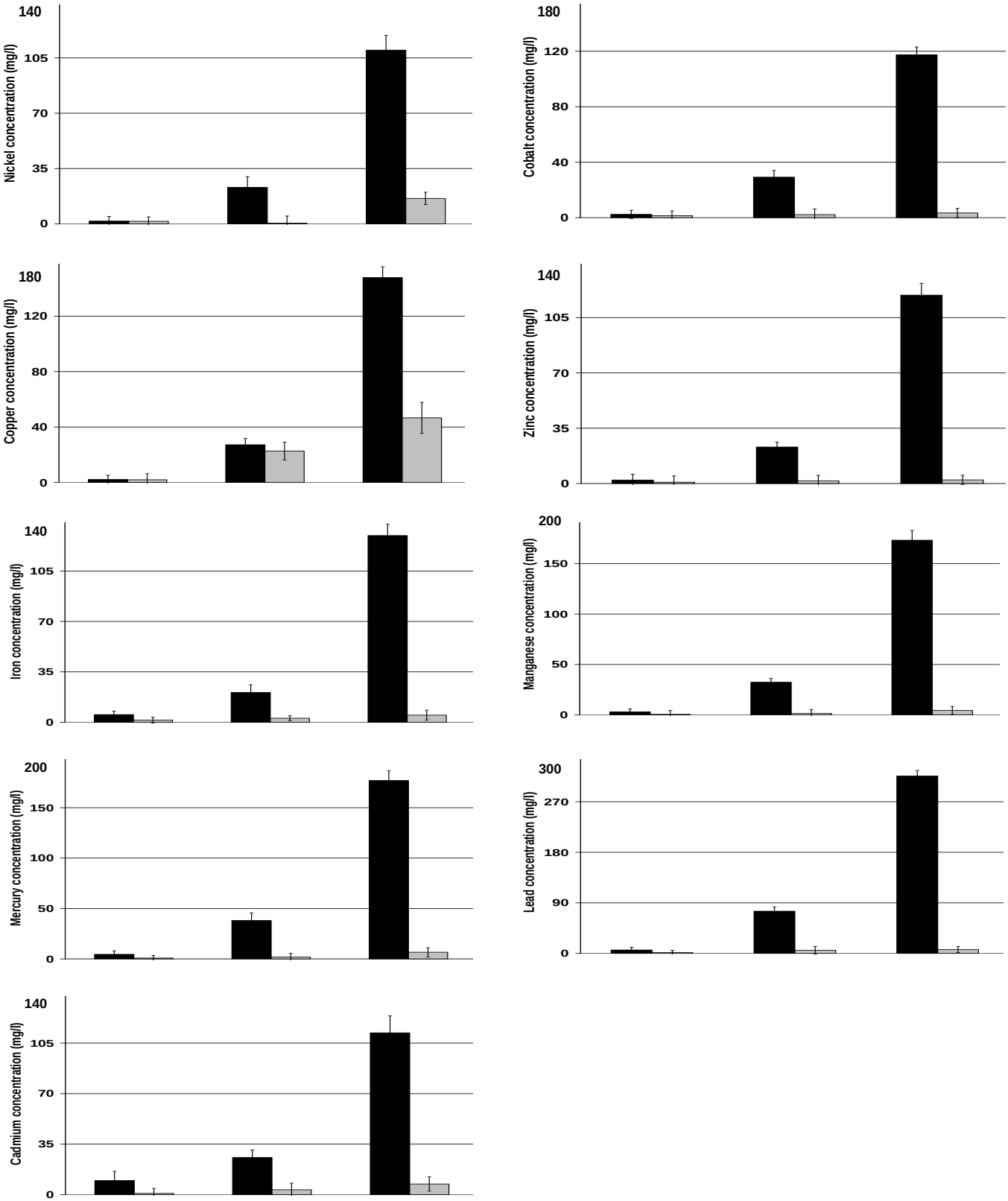


**Figure 3.** Metal bioprecipitation using the biogas produced from an aerobic bacterial culture of the activated sludge against different concentrations of metals as single metal species after 24 h of exposure time. Black columns indicate the initial concentrations while the grey columns indicate the final concentrations. Data are the means of three replicates. Error bars represent the standard errors of the means.

**Table 3.** Metals bioprecipitation from a combined metal solution using the biogas produced during the growth of the bacterial population of the activated sludge on raw domestic wastewater for 24 h. Initial pH was around 6.7 and the final pH was around 9.2. Data are the means of three replicates ± standard errors of the means.

| Metal ion | Initial concentration ($mgL^{-1}$) | Final concentration ($mgL^{-1}$) | Metal removal (%) |
|---|---|---|---|
| $Pb^{2+}$ | 32.6±3.2 | 0.7±0.2 | 97.9 |
| $Zn^{2+}$ | 23.4±2.5 | 3.5±0.3 | 85.0 |
| $Fe^{3+}$ | 21.3±1.3 | 0.3±0.8 | 98.6 |
| $Mn^{2+}$ | 18.5±1.4 | 1.1±1.2 | 94.1 |
| $Ni^{2+}$ | 20.7±2.7 | 1.2±0.8 | 94.2 |
| $Co^{2+}$ | 19.7±1.5 | 0.8±1.4 | 95.9 |
| $Cd^{2+}$ | 32.1±3.6 | 6.1±2.3 | 80.9 |
| $Cu^{2+}$ | 27.8±1.2 | 11.1±1.4 | 60.1 |
| $Hg^{2+}$ | 26.0±1.9 | 4.7±2.5 | 81.9 |

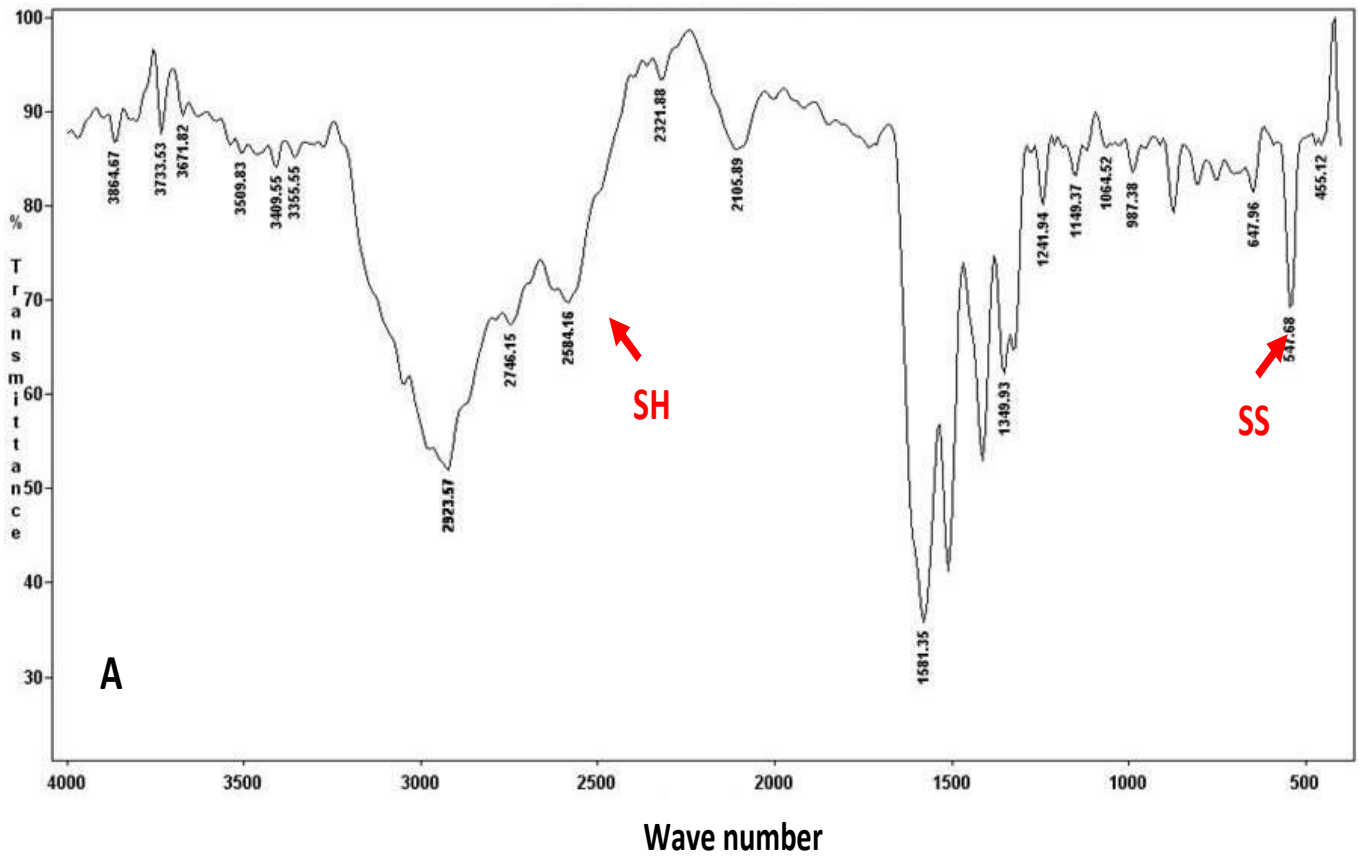


**Figure 4.** FTIR spectra of the single metal precipitates of (A) Mercury; (B) Cadmium and lead and; (C) Copper, zinc, nickel, cobalt, iron and manganese obtained using the culture biogases of the bacterial population of the activated sludge.

headspace gas, with these metals forming insoluble metallosulfur precipitates. Moreover, other studies (Glendinning et al., 2005; Kelly et al., 2006; Lefebvre et al., 2007) have attributed the transformation of soluble metal ions into metal precipitate through the interaction with organothiol compounds produced by some microbial species.

In the bacterial biogas, ammonia as by-product of protein catabolism may be responsible for shifting the pH value of the metal solution toward alkalinity (Table 3).

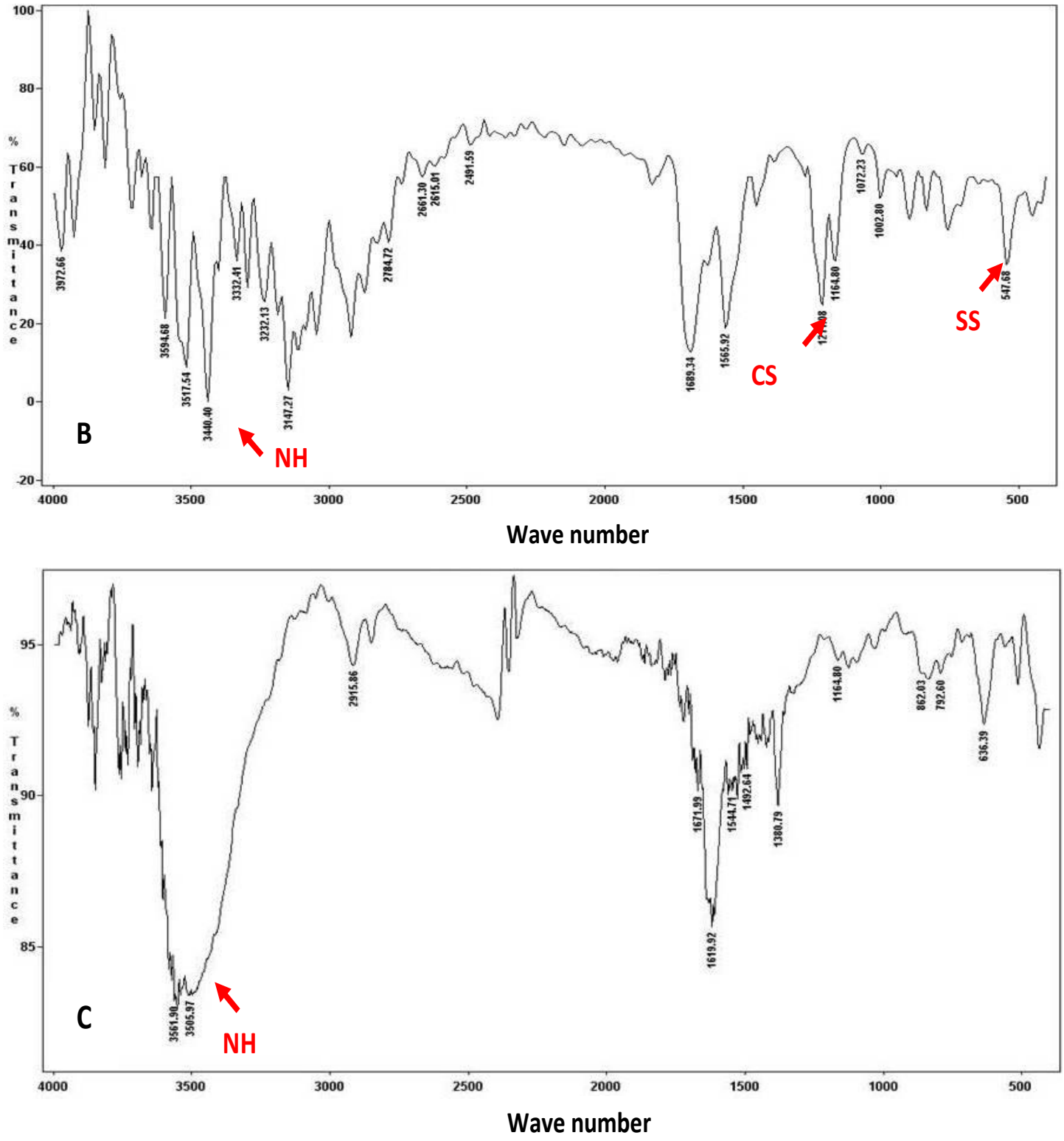


**Figure 4.** Contd.

The biogenic ammonia was assumed to play a significant role in the complexation of some metal ions into nitrogen-based precipitates. This hypothesis is in agreement with our previous study (Macaskie et al., 2007) that showed a

successful recovery of some precious metals from an industrial effluent, based on the complexation of these metals into nitrogen-based complexes, using the biogas produced from *K. pneumoniae* culture. Moreover, the strong affinity of some heavy metals (Cu, Hg, Cd and Hg) to be coordinated with volatile nitrogenous ligands of some cyanobacterial culture-off gases was used successfully to precipitate these metals (Essa and Mostafa, 2011).

## Conclusion

Many volatile compounds are produced naturally during the growth of bacterial population. The headspace gases of the bacterial culture containing volatile ligands were used efficiently to precipitate different heavy metals as sulfur/nitrogen based complexes. Consequently, this bioprocess can be applied for the elimination of heavy metals from contaminated wastes especially the aggressive industrial effluents in a cost effective and eco-friendly process. Moreover, the uncontaminated bacterial biomass can be used safely in different applications.

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