Changes in nutrient profile of soil subjected to bioleaching for removal of heavy metals using *Acidithiobacillus thiooxidans*

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Abstract

Studies were carried out to assess changes in nitrogen, phosphorus and potassium contents in soil during bioleaching of heavy metals from soil contaminated by tannery effluents. Indigenous sulfur oxidizing bacteria *Acidithiobacillus thiooxidans* isolated from the contaminated soil were used for bioremediation. Solubilization efficiency of chromium, cadmium, copper and zinc from soil was 88, 93, 92 and 97%, respectively. However, loss of nitrogen, phosphorus and potassium from the soil was 30, 70 and 68%, respectively. These findings indicate that despite its high potential for removal of heavy metals from contaminated soils, bioleaching results in undesirable dissolution/loss of essential plant nutrients. This aspect warrants urgent attention and detailed studies to evaluate the appropriateness of the technique for field application.
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1. Introduction

Soils contaminated with heavy metals have been a major cause of concern all over the world, as they pose health hazards to living beings and contaminate the environment. Developing effective and low-cost methods to remediate such soils has been the focus of environmental research during the past few decades. Traditional physico-chemical processes available for treatment of contaminated soils are expensive and often do not alleviate pollution hazards. For instance, stabilization/solidification is not very efficient when soil has more than 50% moisture and also presence of organic contaminants affects the process. Moreover, the long-term stability of the solidified/stabilized matrix is still not clear. Vitrification another treatment method is influenced mainly by high moisture and organic content. The application of soil washing another common technique is restricted only to weakly bound material. Thermal treatment suffers due to increased cost when soil has higher water content [1,2]. Though physical containment is least expensive

treatment option but it leaves the pollutants in the site without treatment [2]. In recent times, biologically driven solutions to environmental problems have gained worldwide focus, as they are cost-effective, safe, reliable and sustainable in the long run. Phytoremediation has gained tremendous attention as an *in situ* treatment technique that offers an alternative to the physico-chemical process. However, this process suffers from some limitations such as longer residence time before remediation reaches an acceptable level [3], potential contamination of the food chain, if animals graze on heavy metal contaminated vegetation [4] and low depth of remediation, which need to be overcome. Bioleaching is a method of bioremediation of heavy metal-laden soils, sediment and domestic/industrial sludge [5]. Zagury et al. [6] demonstrated that indigenous iron oxidizing bacteria could be successfully adapted for bioleaching using serial transfer technique from the metal contaminated soil. It is known that metal mobilizing bacteria can be easily enriched from most type of soils and it can be effectively used for solubilization of heavy metals [7,8]. Sulfur oxidizing bacteria has been used employed for remediation of metal contaminated soil by White et al. [9]. Bioleaching of heavy metals from contaminated soil using iron oxidizing bacteria in a semi-continuous reactor has been reported to reduce the leaching time of copper, manganese and zinc [10]. Report of Liu et al. [11] suggest that culture age and temperature play an important role in

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removal of cadmium from soil using *Acidithiobacillus thiooxidans*. Bioleaching has also been applied to remove arsenic from mining soil slurry [12]. Though these reports available in literature confirms the potential of bioleaching as a process to remediate metal contaminated, considerable scope exists to investigate the role of several parameters that influence the process. The present study aims to investigate one of the major aspects of bioleaching, i.e. loss of nutrients during the treatment process.

During bioleaching, a highly oxidizing environment coupled with very low pH levels brings about the dissolution of heavy metals and also the digestion of organic matter [13]. Digestion of organic matter may release 'bound nutrients' into the medium. Loss of nitrogen and phosphorus has been reported while carrying out bioleaching of heavy metals from sewage sludge [14] and soil–sludge mixtures [15]. However, changes in nutrient profile of soil during bioleaching and after soil decontamination have not been reported. This paper assesses the changes in nitrogen, phosphorus and potassium profiles during and after bioleaching of heavy metals from contaminated soil.

2. Materials and methods

2.1. Soil collection and analysis

Contaminated soil was collected from Ranipet, an industrial town in India. Soil samples were manually collected from a depth of 15–20 cm using a plastic scoop, transported to the laboratory in airtight polythene bags and stored at 4 °C.

Soil was air-dried at room temperature and sieved through a 2 mm sieve. pH and electrical conductivity of the soil was measured using 1:2.5 (soil:distilled water) soil slurry. Cation exchange capacity was determined following Rowell [16]. Total Kjeldahl nitrogen (TKN) was determined following APHA [17]. Total phosphorus was determined using the stannous chloride method after acid digestion (HNO_3 and $HClO_4$) of soil. Potassium was determined after ammonium acetate extraction of soil [18] and analyzed using flame photometer (Elico India CL 22D). Sulfate and organic matter content of soil was determined as detailed in Trivedy et al. [18]. To determine the total metal content of the soil, samples were digested with aqua regia [19]. The metals were analyzed using atomic absorption spectrophotometer (Vario-6, Analytik Jena, Germany). Fractionation of heavy metals from soil was done following the method of Tessier et al. [20].

2.2. Isolation and identification of indigenous A. thiooxidans

Indigenous *A. thiooxidans* was isolated from contaminated soil using sulfur medium as detailed in Kumar and Nagendran [21]. The culture was grown on a solid medium [22] to study its colony and growth characteristics. Molecular identification of the isolated culture was done by sequencing the 16S rRNA gene sequences.

2.3. Bioleaching experiments

Batch bioleaching experiments were conducted in 250 ml Erlenmeyer flasks. Fifteen flasks, each with a working volume of 100 ml containing 90% (v/v) of distilled water, 3% (w/v) of contaminated soil, 0.5% (w/v) of elemental sulfur and 10% (v/v) of *A. thiooxidans* (inoculum) were agitated on a rotary shaker at 150 rpm and 30 °C. Likewise, control units having autoclaved soil without *A. thiooxidans* inoculation were also maintained.

pH and ORP were measured once in 2 days during the process using pH meter (Elico, India) and ORP meter (Eutech Instruments, Singapore). pH meter was calibrated using pH 2.0, 4.0 and 9.2 buffer solutions and ORP meter was calibrated using Quinhydrone 255 (Merck Ltd.). At weekly intervals, the contents of three flasks were centrifuged at 3500 rpm for 15 min and the supernatant was filtered using Whatmann No. 42 filter paper. The filtrate was acidified using concentrated nitric acid and analyzed for sulfate, nitrogen, phosphorus and potassium [17]. Heavy metal analyses were carried out using AAS. After filtration, the filter papers retaining soil were air dried at room temperature. The soil was scraped and analyzed for nitrogen, phosphorus and potassium.

3. Results and discussion

3.1. Physico-chemical characteristics of soil

The physico-chemical characteristics of soil are furnished in Table 1. The slightly alkaline soil was rich in nitrogen, phosphorus and potassium that could be utilized by the isolated sulfur oxidizing bacteria. Soil was rich in calcium and magnesium but had low sulfate levels. The organic matter content of soil was also slightly high. From the table, it is also evident that chromium is the major pollutant present in the soil. This is because of unscientific disposal of effluents from a large number of tanneries in the area.

Table 1
Physico-chemical characteristics of metal contaminated soil

Sl. No.	Parameter	Values
1	pH	7.8
2	Electrical conductivity	3.3 ± 0.2
3	Cation exchange capacity	22.6 ± 1.4
4	Total Kjeldahl nitrogen	4862 ± 158
5	Phosphorus	5069 ± 173
6	Potassium	446 ± 17
7	Sulfate	1428 ± 68
8	Organic matter	4.0 ± 0.28
9	Calcium	39,495 ± 1241
10	Magnesium	14,205 ± 547
11	Cadmium	9.1 ± 0.2
12	Chromium	11,810 ± 295
13	Copper	96 ± 4.1
14	Zinc	238 ± 14.5

(Data represent average of five soil samples; ± indicates standard deviation) all values except pH, E.C ($mS\,cm^{-1}$), CEC ($cmol_c\,kg^{-1}$) and organic matter (%) are expressed in mg/kg.

Table 2
Fractionation of heavy metals present in soil used for bioleaching experiments

SI. No.	Heavy metal[a]	F1	F2	F3	F4	F5
1	Cd	3.5 ± 0.1	0.1 ± 0.01	1.7 ± 0.10	1.6 ± 0.1	2.2 ± 0.1
2	Cr	3.9 ± 0.2	65 ± 2.9	9344 ± 387	683 ± 29	1676 ± 78
3	Cu	0.6 ± 0.02	0.7 ± 0.04	4.7 ± 0.2	10.9 ± 0.5	79 ± 3.1
4	Zn	0.9 ± 0.04	17 ± 0.7	36 ± 1.5	78.5 ± 3.4	105 ± 4.7

F1, exchangeable fraction; F2, carbonate fraction; F3, Fe–Mn oxide fraction; F4, organic fraction; F5, residual fraction.

[a] Concentration of heavy metals in soil: Cd, 9.1 mg/kg; Cr, 11,810 mg/kg; Cu, 96 mg/kg; Zn, 238 mg/kg (all values are in mg/kg; data represent average of triplicates).

Table 2 shows the data on fractionation of heavy metals present in soil. From the table, it is evident that the major contaminant chromium was bound mainly to Fe–Mn oxides and it was in very less concentration in exchangeable and carbonate fraction of soil. Cadmium was mainly present in the exchangeable and residual fraction. Copper and zinc were present in the highest concentration in the residual fraction. Concentration of copper and zinc bound to exchangeable and carbonate fractions were low.

3.2. A. thiooxidans

A. thiooxidans isolated from contaminated soil grew well on medium containing elemental sulfur as energy source. The bacterium utilized thiosulfate, as evidenced by the growth of typical small straw yellow colored colonies on the solid medium in 5–6 days. Microscopic examination of the culture revealed the presence of gram negative, rod shaped and motile bacteria. The isolated microorganism was confirmed as A. thiooxidans after classification based on homology of its 16S rRNA gene sequences from the National Center for Biotechnology Information (NCBI) databank by use of BLASTN. The gene sequence was submitted to GenBank with accession number DQ834372. Growth curve of the isolated A. thiooxidans in the sulfur medium over a period of 28 days is shown in Fig. 1.

3.3. pH changes during bioleaching

Changes in pH during bioleaching are shown in Fig. 2(a). During the 4-week experiment, rapid drop in pH was recorded in the first week. From pH 6.6 on day-1, it dropped to 2 within 6 days. On day-18, the pH was <1. The sharp reduction in pH observed during bioleaching is due to the production of sulfuric acid by the activity of A. thiooxidans. The bacteria oxidized elemental sulfur (energy source) leading to soil acidification. In the absence of inoculum in control units, the soil exhibited a tendency to regain its natural alkaline pH. Marginal increase in pH observed in control units indicates that chemical oxidation of sulfur did not occur. A slight increase in pH towards alkaline range observed in control units could be attributed to buffering action and release of substances of basic nature [23]. Though the release of carbonates and other substances of basic nature are known to increase system pH, acid produced by the bacterium appeared to offset the buffering capacity of soil. The final pH of soil in experimental and control units was 0.9 and 7.8, respectively. Villar and Garcia [24] have also recorded sim-

ilar pH profile during bioleaching of metals from anaerobically digested sludge by sulfur oxidizing bacteria.

3.4. Changes in oxidation–reduction potential during bioleaching

Changes in ORP during bioleaching are shown in Fig. 2(b). ORP increased rapidly between day-6 and day-16 and finally it was 612 mV at the end of the experiment. This may be due to sulfur utilization (oxidation), i.e., as the elemental sulfur is oxidized to soluble sulfate, it leads to decreased concentration of free electrons followed by increase in ORP [25]. In control, no significant increase in ORP was recorded. Similar observations on ORP increase with and without addition of sulfur have also been reported by Lombardi et al. [26] during bioleaching of heavy metals from sludge employing A. thiooxidans.

3.5. Production of sulfate during bioleaching

Production of sulfate during bioleaching is presented in Fig. 3(a). Sulfate production increased with passage of time and reached 8.5 g/l on day-28. It was negligible in control units (0.18 g/l). Sulfate production is indicative of oxidation of sulfur added. Oxidation of sulfur is an important step in the process
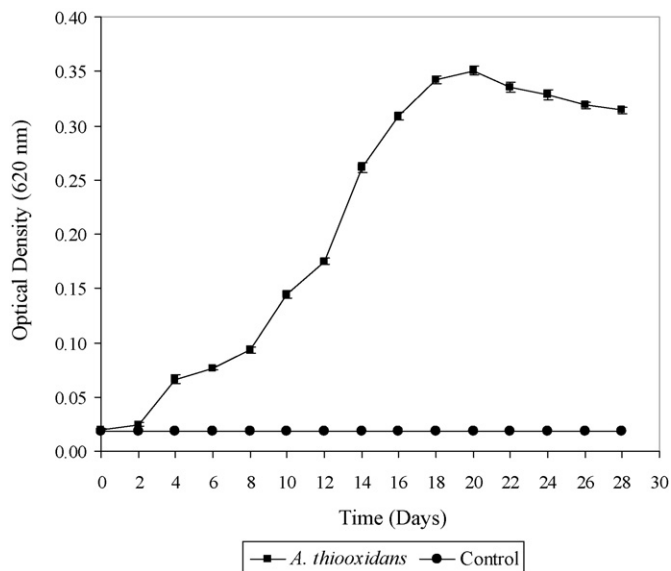

Fig. 1. Growth curve of Acidithiobacillus thiooxidans in sulfur medium (data are average of three growth experiments).
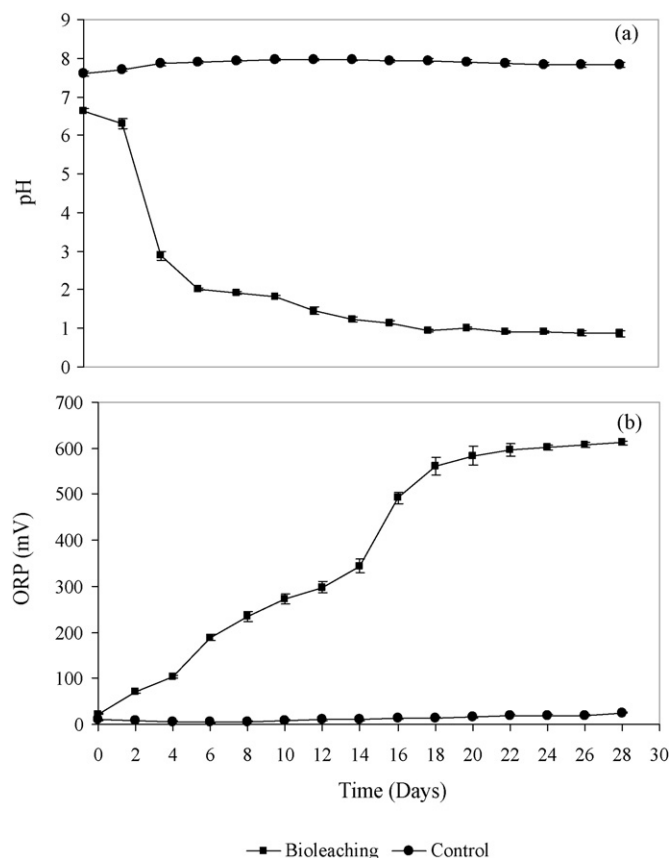
Fig. 2. Changes in soil (a) pH and (b) ORP during bioleaching of heavy metals (data are average of triplicates and bars indicate standard deviation).
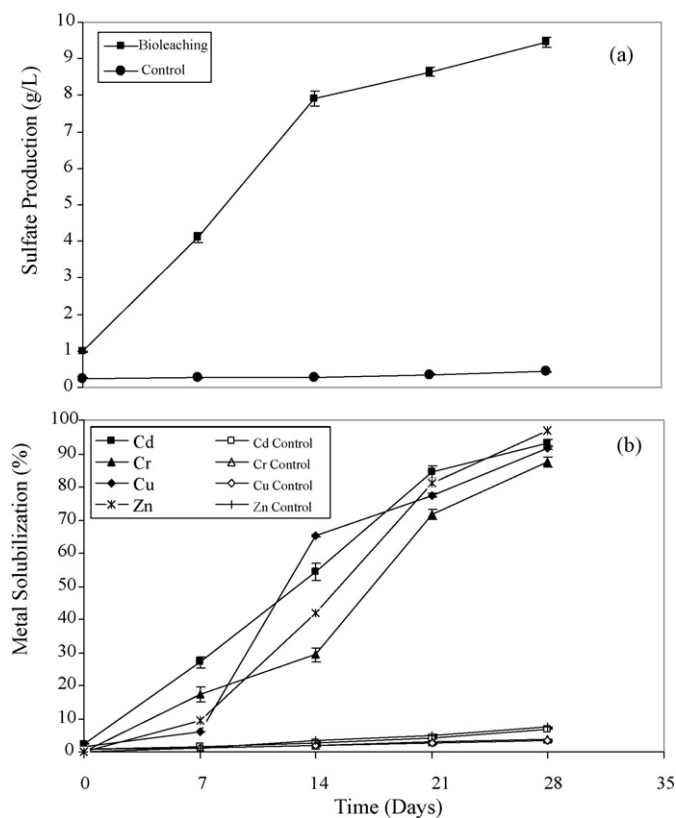

Fig. 3. (a) Sulfate production and (b) heavy metal solubilization from soil during bioleaching (initial concentration of metals (mg/kg): Cd, 9.1; Cr, 11,810; Cu, 96; Zn, 238) (data are average of triplicates and bars indicate standard deviation).

as it results in fall in system pH and produces a bio-oxidative environment that is conducive for removal of heavy metals from solid matrices. It is well known that *A. thiooxidans* first gets adsorbed on to the surface of sulfur by means of van der Waals forces and then oxidizes it [27]. There was steep increase in sulfate production during the first 3 weeks of the study; this could be attributed to the active phase of bacterial growth and consequent higher utilization of sulfur. Slowing down of sulfur production during the latter part of the experiment could be due to the fall in biomass of *A. thiooxidans* triggered by lowering pH [28].

3.6. Heavy metal solubilization during bioleaching

Solubilization of chromium, cadmium, copper and zinc from soil is depicted in Fig. 3(b). Solubilization efficiency of chromium, cadmium, copper and zinc was 88, 93, 92 and 97%, respectively. Solubilization of chromium increased as pH decreased, accompanied by incubation time. In control, solubilization of heavy metals was very low-chromium, cadmium, copper and zinc were solubilized to the extent of 4, 7, 3 and 7%, respectively. Higher solubilization of heavy metals in experimental units compared to control could be attributed to low pH and high sulfate concentrations. It is reported that solubilization of chromium is governed by pH—solubilization being faster at low pH and increasing with time [29]. The solubilization trend in respect of this metal observed in the present study is in con-

formity with the report of these authors. Higher solubilization of cadmium recorded in the present study could be attributed to increasing concentration of sulfate in the system with time [11]. Unlike other heavy metals, copper and zinc exhibited an initial lag phase during the first week. This is because these metals form complexes with organic matter and in this case they were mainly present in the residual fraction of the soil, which are not easily leached out in normal conditions—they require much lower pH [30]. Despite this lag phase, copper and zinc exhibited better solubilization as compared to other metals. This indicates that, once the pH drops below 2, copper and zinc can be easily leached out from soil.

3.7. Changes in nutrient profile of soil during bioleaching

During the first 2 weeks of bioleaching, 1062 mg/kg of TKN was leached out (Fig. 4(a)), which accounted for 22% loss (Table 3) and the corresponding pH was 1.91. This drop in pH was accompanied by further loss of TKN (3436 mg/kg) from soil. 30% TKN loss (see Table 3) was recorded at the end of the process. High TKN content in leachate at the end of 4 weeks of bioleaching could be due to release of nitrogen 'bound' to organic matter in soil under low pH conditions. Loss of TKN may also be attributed to breakdown of soil organic matter and destruction of proteins from microorganisms in acidic environment. The longer the soil remains in such an environment, higher will be the loss of TKN as ammonia [31]. Only 6% leach-
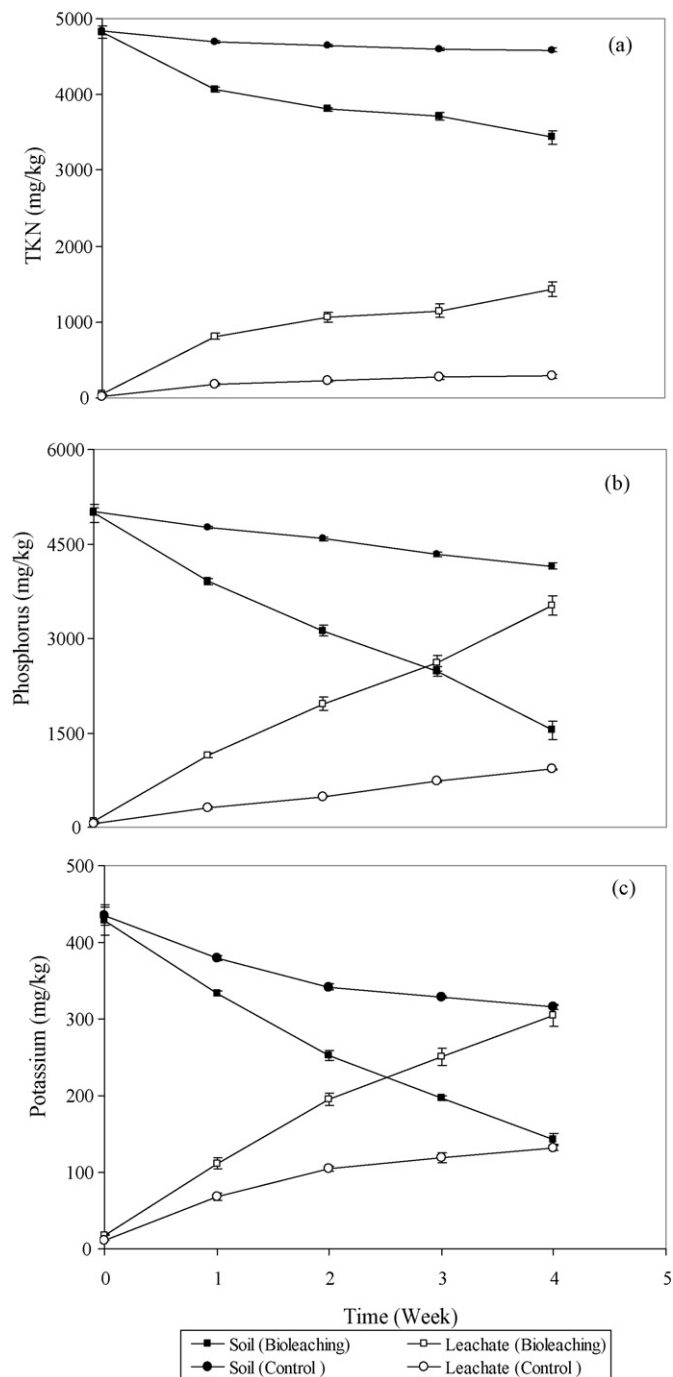
Fig. 4. Changes in (a) TKN, (b) phosphorus and (c) potassium from soil during bioleaching of heavy metals (initial nutrients (mg/kg): TKN, 4862; phosphorus, 5069; potassium, 446) (data are average of triplicates and bars indicate standard deviation).

Table 3
Loss of nutrients from soil during bioleaching

Sl. No.	Nutrients	Loss of nutrients[a] (%)							
		Experimental				Control			
		1st	2nd	3rd	4th	1st	2nd	3rd	4th
1	TKN	16	22	24	30	4	5	6	6
2	Phosphorus	23	39	51	70	6	10	14	18
3	Potassium	25	44	56	68	15	23	27	30

[a] Loss of nutrients at weekly intervals from the experimental soil and control soil during bioleaching. Data are average of triplicates.

Phosphorus leached out from soil was 1961 mg/kg during the first 2 weeks (Fig. 4(b)), accounting for a loss of nearly 40% (Table 3). During subsequent weeks, soil was leached of 3527 mg phosphorus/kg, resulting in 70% loss (Table 3). Phosphorus solubilization was found to be more rapid as pH decreased to 2.0 and below. The control units exhibited 18% loss; the pH in control was slightly alkaline and even at this pH appreciable amount of phosphorus was solubilized. This further testifies that drop in pH is the main reason for solubilization of phosphorus. Highly comparable 76% phosphorus leaching from biosolids during bioleaching of heavy metals using sulfur oxidizing bacteria has been reported by Shanableh and Ginige [33]. In contrast, Tyagi et al. [32] have reported little or no loss of phosphorus during bacterial leaching of heavy metals by *T. ferrooxidans* from sludge in continuously stirred tank reactor and air-lift reactor operated with low retention time. According to Benmoussa et al. [14], loss of phosphorus from the soil during bioleaching could be minimized by maintaining pH < 2 and lowering retention time.

Leaching pattern of potassium from soil during the 4-week bioleaching is presented in Fig. 4(c). Nearly 1/4 (112 mg/kg) of potassium was lost from the soil during the first week itself. Further, as the pH dropped to lower levels, more potassium was solubilized, and at the end of the experiment, soil contained 143 mg potassium/kg (68% loss, Table 3). It is interesting to note that even in control, 30% of potassium was leached out from soil. This indicates that potassium is an easily solubilized nutrient from soil. Highly acidic environment hastened the leaching of potassium. In contrast to the present observations, lower potassium removal has been reported during simultaneous sewage digestion and metal leaching process by Benmoussa et al. [14] and Filali-Meknassi et al. [31]. Difference in experimental design and reactors used by them may account for the variation. Limited literature on loss/leaching of potassium during bioleaching, limits further discussion on this aspect. High potassium content in the leachate of control units (30%) is due to its higher solubility in water.

4. Conclusion

From the foregoing it may be surmised that, despite its high potential for removal of heavy metals from contaminated soils, bioleaching results in undesirable dissolution/loss of essential plant nutrients, viz., nitrogen, phosphorus and potassium. This aspect warrants urgent attention and detailed studies to evaluate

ing of TKN from soil was observed in control. Shanableh and Omar [15] have also observed similar leaching of TKN during bioleaching of heavy metals from soil and sludge mixtures by sulfur oxidizing bacteria. In contrast, there are reports of increase in TKN content after bioleaching of heavy metals from sewage sludge [32]. Such increases in TKN have been related to the use of different reactor-types (continuously stirred tank reactor and air-lift reactor) and bacterial strains used for bioremediation.

the appropriateness of the technique for field application. Studies on minimizing the leaching of nutrients from soil during the treatment are under progress in our laboratory.

Acknowledgement

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