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Bioleaching of copper, cobalt and zinc from black shale by *Penicillium notatum*

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In this study, the recovery of metals from low grade black shale ore was attempted by employing fungal strain, *Penicillium notatum* using different organic wastes as substrates. Maximum recovery of copper (49.29%) and cobalt (53.51%) was found in media containing glucose (standard medium) and molasses as substrate at 28°C after 33 and 30 day of leaching period at 120 rpm. Whereas the maximum concentration of zinc (79.11%) was found in medium containing acidified mango peel as substrate under similar conditions. Extractions were compared with chemical leaching, where leaching up to 54.27% Co and 35.16% Zn were achieved in medium of 1% oxalic acid whereas, copper up to 51.22% extracted in medium containing 1% citric acid. Recovery of metals from this ore has indicated that this low grade discarded ore may be potential source for metals in future prospect.

Key words: Bioleaching, *Penicillium notatum*, organic wastes, organic acids, metals solubilization.

INTRODUCTION

Bioleaching is a process based on the ability of micro-organism (bacteria and fungi) to transform solid com-pounds into soluble and extractable elements, which can be recovered. It represents a 'clean technology' in the mining industries with low cost and capital inputs required as compared to conventional methods that are very ex-pensive for the recovery of metals from low and lean grade ores such as shales and schist as well as increase the environmental hazards (Devasia and Natarajan, 2004). The bioleaching allows the cycling of metals by a process close to natural biochemical cycles reducing the demand for resources such as ores, energy or landfill space. This process has gained importance in a variety of mineral industries. On the basis of interaction between metals and microbes, biohydrometallurgy comprises of different disciplines such as bioremediation, biosorption, bioaccumulation and bioleaching (Devasia and Natarajan, 2004; Xu and Ting, 2009). Microbially metal-extraction

processes are usually more eco-friendly and economical than physico-chemical processes. Several mechanisms may be involved in bioleaching these include (i) cytolysis, (ii) complexolysis, (iii) redoxolysis, and (iv) bioaccu-mulation (Tasa et al., 1997). Compared to bacterial leaching, fungal leaching has the following advantages: (i) ability to grow under higher pH and thus is more sui-table in bioleaching of alkaline solid waste, (ii) a generally faster leaching process with shorter lag phase, and (iii) ability of excreted metabolites (e.g. organic acids) to form complexes with metal ion, thus reducing its toxicity to the biomass.

Hetrotrophs require carbon as energy source to pro-duce organic acid metabolites which include citric, oxalic and gluconic acids during bioleaching (Kucha and Mayer, 1996) and this requirement can be fulfilled by using organic wastes. Acidolysis is the principal mechanism in bioleaching with fungal strains like *penicillium notatum* and *Aspergillus niger*. Agricultural and industrial wastes are among the causes of environmental pollution. Their conversion into useful products may ameliorate the pro-blems they cause. These wastes which include cereals, straw, leaves, seed cake, corncobs, mango peel, rice

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bran etc are highly underutilized in the world especially in Pakistan. In most parts of the country, these materials are mainly used as animal feeds. A large quantity is left on farmlands to be decomposed by microorganism such as bacteria and fungi and produced number of organic acid metabolites like citric, oxalic, malic gluconic acids used for bioleaching of metals (Wainright, 1992).

The main concern of the present research work was to explore the ability of microbes like *Penicillium notatum* to extract metals like Cu, Zn and Co from black shale using different organic wastes as substrates.

MATERIALS AND METHODS

All chemicals and reagents used in the present study were of analytical grade and mainly purchase from Sigma-Aldrich Chemical Company, USA.

Black shale sample

A representative black shale ore sample was obtained from Pakistan Institute of Engineering and Applied Sciences (PIEAS), Islamabad, Pakistan. It was alkaline (pH 7.3) in nature, insoluble in water. The sample was oven dried ground to $\leq$ 200 mesh size by ASTM sieving machine. Elemental analysis was carried out by atomic absorption spectrometry (Perkin Elmer, Analyst 300): Raw sample mainly composed of (mgL^{-1}) Fe 3761.00, Cu 39.07, Co 18.26, Zn 31.88 and Ni 8.25.

Fungal strain and growth conditions

P. notatum was isolated from rotten oranges then cultivated for purification on slants of PDA media (3.9%) described by Bousshard et al. (1996). It was incubated (Incubator, Sanyo, Germany) for 72 h at 28°C to produce an adequate number of spores. The spores were counted using Petroff-Hausser counting chamber. For growth in liquid medium, the culture medium consisted of (g/L): KH$_2$PO$_4$, 5.0; NH$_4$NO$_3$, 2.0; (NH$_4$)$_2$SO$_4$, 4.0; MgSO$_4$.7H$_2$O, 0.2; peptone, 2.0; trisodium citrate, 2.5; yeast extract, 1.0 and made volume up to 1000 mL with distilled water (Bhatti et al., 2007). Nine sets of 250 mL flasks were prepared containing 100 mL of liquid medium each in triplicate samples. Medium in each flask was autoclaved at 121°C for 15 min. After sterilization, 5% of given substrate was added in each flask then inoculated with 1 mL of *P. notatum* spore suspension as inoculum (approximately 1.8 $\times$ 10^6 spores mL^{-1}). All the flasks were sealed with removable cotton and incubated in an orbital shaker (Gellen Kamp, England) at 28°C at an operating speed of 120 rpm for 15 days.

Chemical leaching of metals

To determine the effects of different organic acids on the extraction of metals from black shale, chemical leaching experiments were carried out with citric, oxalic, malic and tartatic acids with different concentrations. Four different concentrations of organic acids 0.05% (w/v), 0.1% (w/v), 0.5% (w/v) and 1% (w/v) with 1% (w/v) of black shale residue (pulp density) in triplicate were subjected to shake flasks treatment for the period of 30 days. The pH was noted during the leaching period after every 2nd day. Supernatants were withdrawn at the end, filtered and analyzed for metals dissolved in each sample by atomic absorption spectrophotometry (Perkin Elmer, Analyst 300).

Pretreatments of substrates

Before the addition of substrates in the media, substrates were subjected to pretreatment process. Glucose was used as substrate for medium 1. Cane mollases were obtained from local sugar industry. A diluted (50%) solution of mollases was used for medium 2 as substrate. For the experiment with agricultural wastes like mango peel, rice bran and seed cake, these were first oven dried, subjected to fine grinding, weighed and used for media 3, 5 and 7 respectively and for media 4, 6 and 8, these agricultural wastes were subjected to soaking treatment in sulphuric acid of pH 2 for 24 h then weighed and added in the respective media where as control medium contained no any substrate under the same conditions (Table 1).

Leaching of the shale residues with organic acids produced by *P. notatum*

Supernatant liquor of microbial culture was collected after the cellular growth of 15 days, sterilized, centrifuged (8000 rpm for 10 min at 15°C) then filtered to remove solids biomass before HPLC analysis for the determination of organic acid metabolites. Supernatants containing organic acid metabolites used to leach the metals from shale residue. Then 1% (w/v) of pulp density was added in each medium of flasks and incubated on an orbital shaker to keep everything in homogeneous slurry form at 28°C and 120 rpm for 36 days. Samples were collected every 3rd day of leaching period, filtered then subjected to atomic absorption analysis for the estimation of metals.

Analysis of organic acids

Analyses of metabolites (e.g. citric, oxalic, tartaric and malic acids) of all media were performed by following the modified HPLC method as described by Escobal et al. (1996). After centrifugation and filtration, samples were vortexed before HPLC analysis. The mobile phase consisted of 0.25% of acetic acid, was filtered and sonicated to remove if any of the suspended particles. An HPLC (Sykam GmbH, Kleinostheim, Germany) equipped with S-1121 dual piston solvent delivery system and S-3210 UV/VIS diode array detector and software package for data acquisition was used. A 20 µL of filtered sample was injected in to an analytical Hypersil (Thermo Hypersil, GmbH, Germany) ODS reverse phase (C$_{18}$) column (250 $\times$ 4.6 mm; 5 µm particle size) fitted with a C$_{18}$ guard column. The chromatographic separation was performed by isocratic elution of the mobile phase at a flow rate of 1.0 mLmin^{-1} at 30°C. Detection was performed at wavelength of 254 nm. Organic acids were identified by comparing the retention times and quantified on the basis of peak area percent of the unknowns with those of pure standards of oxalic, citric, tartaric and malic acids. The peak areas were recorded and calculated by a computer with, chromatography data acquisition and integration software (SRI Instrument, Torrance, California, USA).

Metals analysis

Samples collected on every third day of leaching period, were subjected to metals analysis by atomic absorption spectrophotometry (Perkin Elmer, Analyst 300). At the end of leaching period of each medium, residue samples were washed with water three times then oven dried. Residues were subjected to wet digestion process by using nitric acid and hydrogen peroxide as described by Environment Canada (1990).

Table 1. Concentration of organic acid in fermented media after 15 days of growth of *P. notatum* (before leaching as well as after leaching).

Medium no.	condition	pH of fermented media	Organic acids (%) w/v			
			Citric	Malic	Oxalic	Tartaric
1	Before leaching	3.2	0.69 ± 0.035	0.99 ± 0.05	0.01± 0.001	ND
1	After leaching	7.9	ND	< 0.01	ND	ND
2	Before leaching	5.4	0.47 ± 0.025	1.32 ± 0.03	0.43 ± 0.02	0.05 ± 0.001
2	After leaching	8.1	0.02 ± 0.00	ND	ND	ND
3	Before leaching	4.0	0.12 ± 0.005	0.07 ± 0.004	ND	ND
3	After leaching	7.5	ND	ND	ND	ND
4	Before leaching	3.6	1.11 ± 0.04	0.89 ± 0.04	0.19 ± 0.009	0.11± 0.004
4	After leaching	7.9	ND	0.01± 0.00	ND	ND
5	Before leaching	4.3	0.14 ± 0.005	0.12 ± 0.003	ND	ND
5	After leaching	7.6	ND	ND	ND	ND
6	Before leaching	3.9	0.71 ± 0.03	0.92 ± 0.02	ND	0.04 ± 0.001
6	After leaching	7.9	ND	< 0.01	ND	ND
7	Before leaching	4.7	0.06 ± 0.00	< 0.01	ND	ND
7	After leaching	7.9	ND	ND	ND	ND
8	Before leaching	3.0	0.52 ± 0.01	0.73 ± 0.03	ND	ND
8	After leaching	7.2	< 0.01	< 0.01	ND	ND

Values are mean ± SD of duplicate samples analyzed individually in triplicate.

Statistical analysis

All experiments of the samples before leaching and after leaching were performed in triplicate and the results were reported as mean ± SD (Steel et al., 1997).

RESULTS AND DISCUSSION

Physical appearance of black shale ore was black in color and nature was found alkaline. It was insoluble in water. The coloring material in individual shales might be carbon, hydrocarbon or finely divided iron sulfide.

Chemical leaching

For the optimization of conditions for acids, chemical leaching tests were conducted using oxalic, citric, tartaric and malic acid used in different concentrations (0.05, 0.1, 0.5 and 1%) with fixed pulp density (1%). It was found that metals solubilization increased as the concentration of acid was increased which resulted in the increase in pH of media due to the consumption of proton. Optimum recovery of copper (51.22%) was achieved in case of citric acid (1%). Tartaric acid has least affinity for copper (38.19%) whereas oxalic and malic acid showed the same effect on copper extraction that was 41 and 41.69%, respectively. It means that copper has more affinity for citric acid than other acids. The concentration of copper was 44.18% in medium of 0.5% citric acid. Copper concentration became 51.22% by doubling the acid concentration. With respect to chelating capacity of acids,

citric acid chelated copper more affectively than other acids. Stability constants for copper indicated the tendency of this metal to be chelated by citric acid. Thus, it could be postulated that copper citrate complex might generate in the solution as copper and citric acid interacts as shown in Figure 1a.

Zinc recovered maximum in case of oxalic acid (35.16%) than in citric (14.49%), malic (12.58%) and tartaric acid (08.72%). When acid concentration increased, the affinity of zinc was also increased towards oxalic, tartaric, malic and citric acid (Figure 1b). The affinity of zinc towards oxalic acid might be due to its stronger acidity as compared to other acids and oxalic acid has lower pH as compared to other acids. So, the availability of H^+ is directly proportional to the dissolution of zinc as reported by Burgstaller et al. (1992). Less affinity of zinc towards citric acid might be due to the formation of $Zn_3 Cit_2$ complex which is easily precipitated.

Affinity of cobalt has been found greater towards oxalic acid (54.27%), followed by citric (38.99%), malic (38.17%) and tartaric acid (29.30%). Cobalt solubilization also increased as the concentration of organic acids increased. The solubility trend of cobalt has shown in Figure 1c.

Production of organic acids by *P. notatum*

During the growth study of fungal strain under shake flask treatment conditions, tiny beads of light yellow color were appeared in large number during growth period. Size and number of beads increased as the time progressed. The appeared hyphae were branched, scale like with knobby
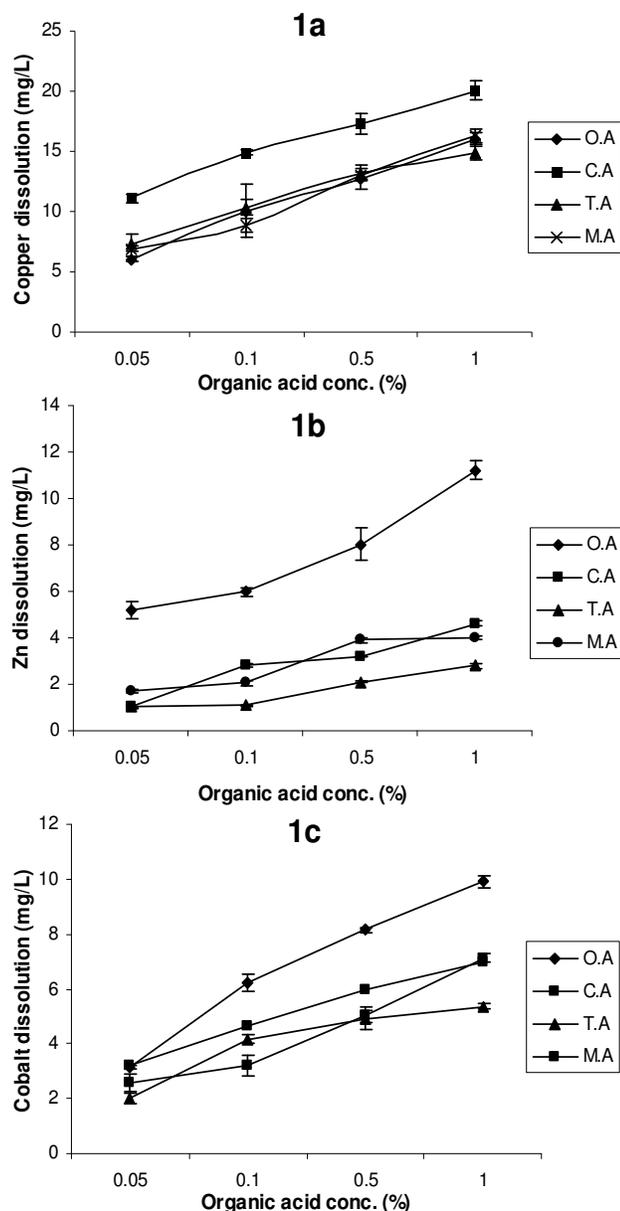
Figure 1. A plot of chemical leaching of metals by organic acids after 30th day (a) copper (b) zinc (c) cobalt. O.A = oxalic acid, C.A = citric acid, M.A = malic acid and T.A = tartaric acid.

ends. On the condensed mass, small hyphae appeared and the color of hyphae was changed from light yellow to bluish green at the end of the growth period. This bluish green color was of conidia because the hyphae of penicillium are colorless in the inoculated flasks (Murad et al., 2003). At the end of growth period, decrease in pH due to organic acid production was observed in the range of 3.0-5.4 in all inoculated flasks except control flask contained no substrate. Maximum biomass production (5.71 %) was noted in the medium having acidified mango peel as substrate followed by the medium having glucose (4.71%), acidified seed cake (4.58%), molasses (3.81%)

and acidified rice bran (3.78%) as clear from Figure 2. High yield of biomass (wet) production is complementary to the acids production. Microbial oxidation of substrate is an acid producing system. During the growth studies of *P. notatum*, substrate undergoes microbial oxidation which results in production of citric, oxalic, tartaric and malic acids that play fundamental role in the environmental mobility of metals (Table 2) (Dronawat et al., 1995; Sayer and Gadd, 2001). Some of these acids act as complexing agent with 2 or more electron donors for metals. Citric, oxalic and tartaric acid have chelating properties and have more affinity for Cr, Ni and Zn in the soil (Elliott and Shastri, 1999; Wasay et al., 2001). To investigate the effectiveness and utilization of organic acids in metal solubilization during leaching process, organic acids produced in all media collected before leaching (After 15 days of growth period) as well as that of sample collected on 36th day of leaching period were analyzed by HPLC. The HPLC data of organic acids are shown in Table 2. It is obvious from the HPLC data that major acid produced by *P. notatum* was citric acid which is followed by malic acid in all analyzed samples after 15 days of incubation. However, oxalic acid produced only in medium 1, 2 and 4 whereas, tartaric acid found only in medium 2, 4 and 6. Some of these acids are good metal chelators which can form complex with metal ions. Organic acid is called chelator if it has two or more electron donor groups. Hence one or more rings are formed and then the organic acid can be termed as chelating agent and resulting complex is termed as metal chelator (Denevre et al., 1996).

Bioleaching of metals

After estimation of organic acid metabolites in supernatants of all media, pulp density (ore concentration) was added in order to determine the effectiveness and enhancement of metals extraction by organic acids. In case of control medium, increased pH (6.1) was noted at the end of the growth period due to the lack of carbon source for the growth of the microbe indicating no production of organic acids. When pulp density (ore sample) was added, again increase in pH was observed that might be due to slight solubilization of basic minerals in the medium. Final pH of the medium approached to 7.2 at the end of the leaching period. Small amount of copper (4.22%), zinc (3.42%) and cobalt (5.31%) was recovered in control sample (Figure 3a). The trend of pH during the leaching period of metal solubilization in the medium containing glucose is shown in Figure 3b. It is clear from Figure 3b that as the leaching process goes on; pH was found to be increased.

At the beginning of the growth period of 15th days, pH of medium was 5.8 that dropped to a value of 3.2 up to 15th day. When ore was added in the supernatant, pH again went on increasing as the leaching period of 36th days started. It approached to 7.9 at the end of leaching period which indicated the organic acids utilization in
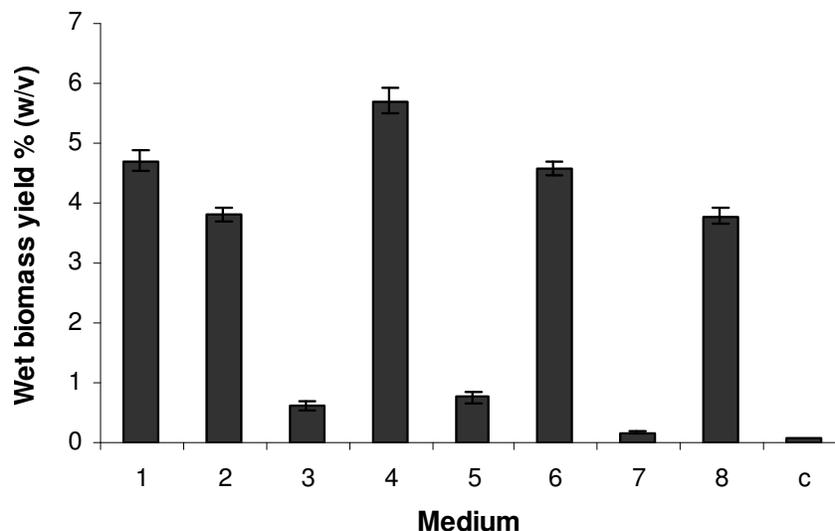
**Figure 2.** Biomass production by *P. notatum* using different substrates.

Table 2. Substrates and pretreatments.

Medium no.	Substrate	Pre-treatment
1	Glucose (5% w/v)	Filtration
2	Molasses (5% of 50% v/v diluted)	Autoclaving (120°C, 5min)
3	Mango peel (5% w/v)	Grinding/ Autoclaving (120°C, 5min)
4	Mango peel (5% w/v)	Grinding/ Sulphuric acid (pH 2)
5	Seed cake (5% w/v)	Grinding/ Autoclaving (120°C, 5min)
6	Seed cake (5% w/v)	Grinding/ Sulphuric acid (pH 2)
7	Rice bran (5% w/v)	Grinding/ Autoclaving (120°C, 5min)
8	Rice bran (5% w/v)	Grinding/ Sulphuric acid (pH 2)

metals dissolution. A significant amount of copper and zinc up to 49.30 and 53.36% was solubilized on 33rd day of incubation which indicated that glucose has very good potential for copper and zinc. Significant solubilization of copper and zinc might be due to the acidic environment of the medium and chelating capacity of the organic acids. Recovery of cobalt was 48.58% of the original value on 30th day of incubation. After 30th day, irregular trend has been observed in cobalt concentration that might be due to the lack of complexation reaction between metals and organic acids (Zhang et al., 2001) and soluble cobalt started to decline. As the *P. notatum* filtrates can solubilize metals more efficiently than commercial organic acids, the pH of medium 2 (Figure 3c) dropped to a value of 5.4 on 15th day of growth period due to organic acids production in the medium.

This low pH approached to a value of approximately 8.1 on 36th day of leaching period due to acids utilization in complex formation with metals in the ore matrix but the copper concentration noted on 30th day was significantly lower (40.80%) than medium 1 (Figure 3b). Maximum recovery of cobalt (53.51%) was noted in the medium

containing molasses as compared to other media. Where as significant amount of zinc (68.54%) was extracted on 30th day of leaching period. In medium 3, mango peel with no pretreatment (Figure 3d), the amount of copper was solubilized up to 14.92% on 30th day of leaching period. After 27th day, decline in copper value was noted. In this medium satisfactory concentration of zinc (24.62%) was found whereas cobalt extracted in lower amount on 30th day that was 16.32%. Solubilization of these metals has resulted in increased pH of that medium from 4.0 to 7.5 up to 36th day of leaching period. Comparatively less solubilization of metals in this medium might be due to the deficiency of available substrate for the fungus. Cellulose and lignin are the main components of agricultural wastes and very hard to break down by microbes. In these circumstances, sulphuric acid has been utilized for the hydrolysis of these chains as in medium 4 (Figure 3e) in which mango peel was subjected to pretreatment by sulphuric acid (pH 4). Maximum amount of copper (46.15%), zinc (79.11%) and cobalt (27.55%) was observed on 27, 33 and 30th day of leaching study. As indicated in Figure 3e, highest amount of zinc of all media
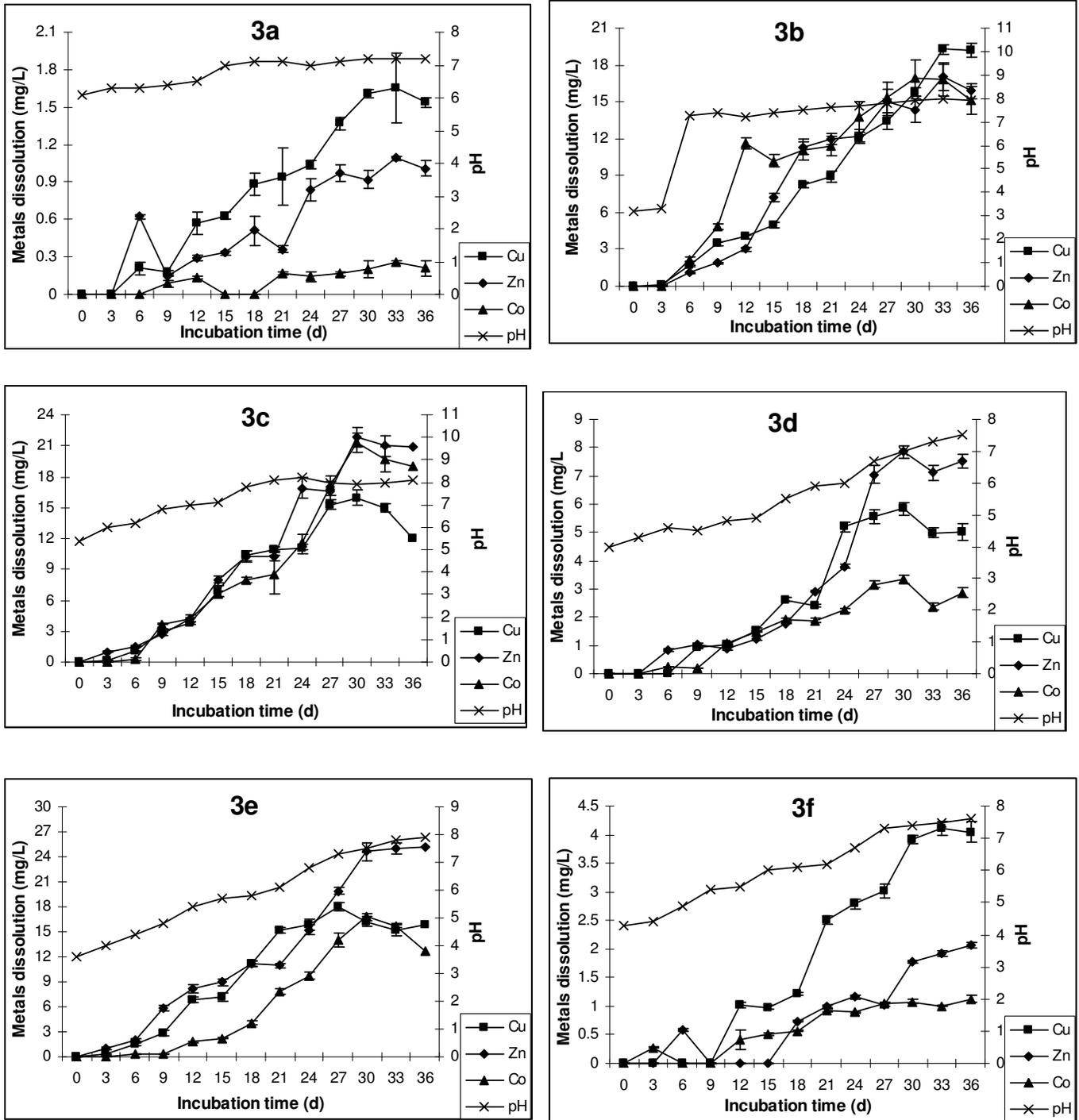
Figure 3. Bioleaching of metals in control medium (a), medium 1 (b), medium 2 (c), medium 3 (d), medium 4 (e), medium 5 (f), medium 6 (g), medium 7 (h) and medium 8 (i).

was found in this medium but this medium also has good potential for copper and cobalt recovery that might be due to the pretreatment effect of substrate to promote the hydrolysis process and enhance organic acid production. Thus, metals solubilization up to 27, 33 and 30th day and then decline in copper, zinc and cobalt value was noted.

Maximum solubilization of these metals has resulted in increased pH of the medium from 3.6 to 7.9 up to 36 d of leaching as depicted in Figure 3e. Another experiment was conducted by using seed cake as substrate with and without acid pretreatment in medium 6 and 5 respectively as illustrated in Figures 3g and 3f. In medium 6,
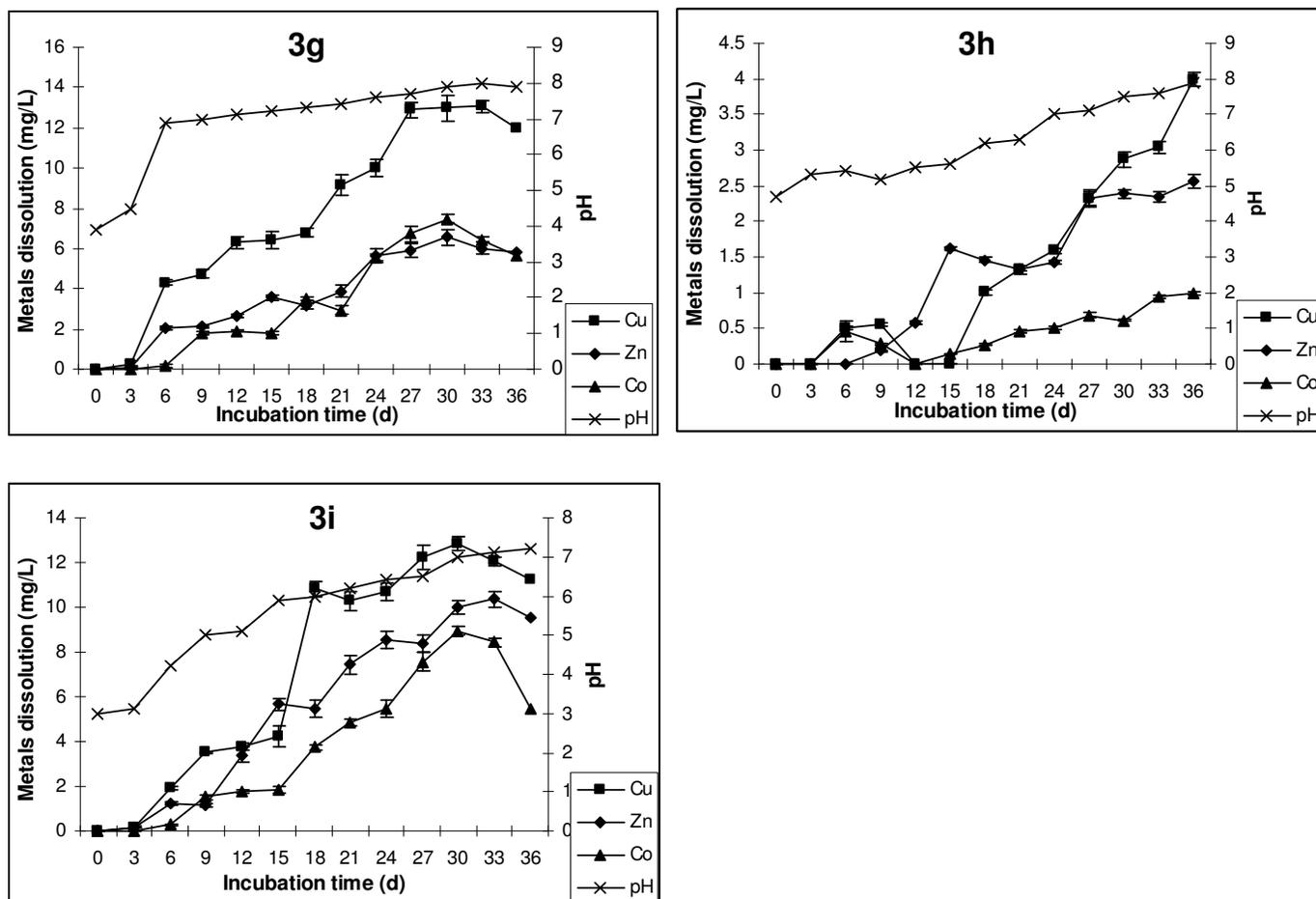
Figure 3. continue.

maximum concentration of all metals observed on 33 and 30th day of leaching study.

Satisfactory amount of copper (33.40%) and zinc (20.55%) was noted on 33 day of leaching and maximum recovery of cobalt (23.06%) was found on 30th day of leaching study whereas medium 5 did not work well due to less growth of microbe in this medium and metals recovery is also not satisfactory as compared to samples treated with acid. The maximum solubilized copper, zinc and cobalt was not more than 10.52, 06.49 and 11.01%, respectively (Figure 3f). As the medium 4, 6 and 8 contain substrates that have sufficient amount of polymeric glucose as cellulosic material, when these substrates were soaked in sulphuric acid then cellulosic material undergo degradation resulted in more free glucose molecules for utilization by microbes as energy source. The efficiency of metals solubilization pronounced to a greater value and show very good potential for copper, cobalt and zinc solubilization as illustrated in respective Figures 3e, 3g and 3i but minimum concentration of zinc (06.49%) found in medium 5 that were not acidified. Medium 7 that contain rice bran as substrate, the

results are shown in Figure 3h. Very poor recovery of metals has been found in case of medium 7 containing rice bran without acid pretreatment. Approximately, 10.21, 08.03 and 10.90% of the copper, zinc and cobalt respectively were solubilized up to 36th day of leaching experiments as depicted in Figure 3h. Its pH decreased to a value of 4.7 on 15th day of growth period. When ore sample was added to it, pH started to go on increasing and reached to a value of 7.9 on 36th day of leaching study.

When rice bran was treated with acid then it showed good potential as substrate utilized by microbes and recovery of metals are considered reasonable as compared to medium containing substrate that was not subjected to acidification. Maximum solubilized copper, zinc and cobalt in this medium was about 32.84, 32.47 and 28.04%, respectively (Figure 3i). It is noteworthy that rice bran medium initial pH was 3.0 at the end of the growth period which indicated the privileged culture growth of microbe resulted in better potential of this media as substrate that contained mostly of lignin and cellulosic material and *P. notatum* can not consume this as

substrate without acid treatment.

Mass balances

Finally, analysis of residual solids after leaching was carried out to perform a mass balance for copper, zinc and cobalt. The amount of copper was determined in the residual solids and then added to the maximum amount of the copper, zinc and cobalt that were solubilized in the supernatant. This was then compared to the initial amount of the copper, zinc and cobalt in the raw sample. On the average, it was found that some of the copper (31.09%), zinc (11.52%) and cobalt (21.48%) have not been accounted for. It might be lost during sample preparation process like filtration in which some of the undissolved salts might adsorbed on the surface of the filter paper etc.

Conclusions

It was concluded from this research work that recovery of metal constituents from shale residue is feasible. In this study *P. notatum* exhibited a good potential for generating varieties of organic acids effective in metal solubilization. Organic acid effectiveness enhanced when sulphuric acid was added to the medium. Different food and agricultural wastes were evaluated with the results of a maximum of 49.30% for copper, 79.11% for zinc and 53.51% for cobalt dissolution. Pretreatment of agricultural wastes have made the process more cost effective and more efficient. Among these wastes, mollases and mango peel showed good potential than seed cake and rice bran and acceptable results were achieved but the results of acidified seed cake and rice bran are also satisfactory.

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REFERENCES

Bhatti HN, Rashid MH, Nawaz R, Asgher M, Perveen R, Jabbar A (2007). Optimization of media for enhanced glucoamylase production in solid state fermentation by *Fusarium solani*. Food Technol. Biotechnol. 45(1): 51-56.

Bousshard PB, Bachofen R, Brandl H (1996). Metal leaching of fly ash from municipal waste incineration by *Aspergillus niger*. Environ. Sci. Technol. 30: 3066-3070.

Burgstaller W, Strasser H, Wbking H, Schinner F (1992). Solubilization of zinc oxide from filter dust with *Penicillium simplicissimum*: Bioreactor leaching and stochiometry. Environ. Sci. Technol. 26: 340-346.

Denevre O, Garbaye J, Botton B (1996). Release of complexing organic acids by rhizosphere fungi as a factor in Norway spruce yellowing in acidic soils. Mycol. Res. 100: 1367-1374.

Devasia P, Natarajan KA (2004). Bacterial leaching: Biotechnology in mining industry, General article, Resonance, pp. 27-34.

Dronawat SN, Svihla CK, Hanley TR (1995). Production of gluconic Acid by *Aspergillus niger*, Appl. Biochem. Biotechnol. 51(2): 347-354.

Elliott HA, Shastri NL (1999). Extractive decontamination of metal-polluted soils using oxalate. J. Water, Air, Soil Pollut. 110: 335-346.

Environment Canada (1990). Service de conservation et protection Region Quebec Methode d'anaiyse des metaux dans sediments, les boues et les sols, pp. 45-47

Escobal A, Gonzalez J, Iriondo C, Laborra C (1996). Liquid chromatographic determination of organic acids in txakoli from Bizkaia. Food Chem. 4(58): 381-384.

Kucha H, Mayer W (1996). Geochemia skał zmineralizowanych, in: Monografia KGHM Polska Miedz SA., Lubin, pp. 237-247.

Murad A, El-Holi K, Al-Delaimy S (2003). Citric acid production from whey with sugars and additives by *Aspergillus niger*. Afr. J. Biotechnol. 2(10): 356-359.

Sayer JA, Gadd GM (2001). Binding of cobalt and zinc by organic acids and culture filtrates of *Aspergillus niger* grown in the absence or presence of insoluble cobalt or zinc phosphate. Mycol. Res. 105: 1261-1267.

Steel RGD, Torrie JH, Dickery D (1997). Principles and procedures of statistics. A biomaterial Approach, 3rd Ed. McGraw Hill Book Co. Inc., New York, USA.

Tasa A, Vourinen A, Garcia O, Tuovinen O (1997). Biologically enhanced dissolution of Pyrite rich Black shale concentrate. J. Environ. Sci. Health, A32(9 & 10): 2683-2695.

Wainright M (1992). An introduction to Fungal Biotechnology. John Wiley & Sons Ltd., West Sussex, England, pp. 36-40

Wasay SA, Barrington SF, Tokunaga S (2001). Organic acids for the in situ remediation of soils polluted by heavy metals: soil flushing in columns. Water Air Soil Pollut. 127: 301-314.

Zhang T, Zhang Z, Zhang D, Zhang C (2001). Enhancement of biooxidative leaching of copper sulfide minerals by using ultrasonic wave treatment, Guocheng Gongcheng Xuebao, 1(3): 315-317.