Bioavailability of Aluminum from Black Shale Using Acidic Metabolites of Heterotrophs

[1]Fozia Anjum, [2]Shazia Anwer Bukhari, [3]Tajnees Pirzada, [3]Mir Munsif Ali Talpur, [4]Mahmood-ur Rahman and [1]Shahzad Ahmad Shahid Chatha,

[1]*Department of Chemistry, Govt. College University, Faisalabad-38000, Pakistan.*
[2]*Department of Biochemistry, Govt. College University, Faisalabad-38000, Pakistan.*
[3]*Department of Chemistry, Shah Abdul Latif Bhitai University (SALU), Khairpur. Pakistan.*
[4]*Department of Bioinformatics and Biotechnology, Govt. College University, Faisalabad-38000, Pakistan.*
bukhari.shazia@yahoo.com; shaziabukhari@gcuf.edu.pk*

(Received on 29th December 2015, accepted in revised form 28th September 2017)

Summary: Aluminum was bioextracted from black shale using *Aspergillus niger* and *Penicillium notatum* as acidophiles. Wet chemical and electron dispersive X-ray analysis (EDX) showed the presence of iron, silicon, aluminum, titanium, copper, sulphur, phosphorus, chlorine, potassium, sodium, calcium, carbon and oxygen in the original shale. A detailed investigation of the leaching process with these microorganisms was conducted. Microbes were tested for acid production and leaching capabilities of aluminum from black shale. Different organic wastes were evaluated as substrates by microbes. Moreover, citric acid generated by fungi could be an important leaching agent acting in the dissolution of aluminum. Aluminum dissolution with *A. niger* was better than those with *P. notatum*. Maximum recovery of aluminum (65.71%) was found in an *A. niger* medium containing acidified mango peel substrate at 28°C after 36days of leaching at 120 rpm. The results obtained with chemical leaching when using different concentrations of organic acids showed that dissolution of aluminum was much higher in citric and oxalic acid than other acids. Besides acidic metabolites production, microbiological activities play an important role in contribution of metals extraction. In future scenario, these discarded ores might be a potential source of metals.

Keywords: Bioleaching, *Penicillium notatum*, *Aspergillus niger*, Organic wastes, Organic acids, Aluminum dissolution.

Introduction

Bioleaching is the recovery of metals from poor ores and mineral concentrates by the exploitation of microorganisms. Demolitions as well as configurational changes in the earth's crust have been taken place since the life began on the earth. Early miners used microbial activity to leach copper from ore as well as rocks. Microbe assisted metals recovery from ores, electronic wastes and used catalysts have been practiced for decades. In recent years, there has been a strongly growing minerals biotechnology industry to extract metals [1-4].

Microbial technology is continually advancing to exploit low grade ore deposits, as high grade surface mineral deposits are worked out. There is an urgent need of time to recover metals from the copious low grade ores. Low grade ores remain important for the future supply of metals as they contain the bulk of known metals like copper, aluminum, cobalt, nickel and iron etc. The traditional hydrometallurgy-based metals resurgence methods are now less beneficial and companies are looking forward to discover new methods to recover the lingering lower grade ore deposits. Bioleaching has advantages in the withdrawal of metals from many low grade ore deposits. Such as a considerable amount of copper and aluminum have been recovered microbialy from low grade ores and plunks that have been left behind from preceding mining operations. [5-7].

Bioleaching is an alternate method for the extraction of metals from their ores that are in very low amount and this method is now using many industries. Thus; aluminum, copper, nickel and zinc metals are easily and efficiently recovered by this process [8-10]. Bioleaching technology has created great interest due to their higher efficiency, lower costs and small industrial requirements [11].

Aluminum is a metal with general large scale use in modern industry. The world production of aluminum is based on the well-known Bayer's process. However, only high grade ores can be treated by this method. The use of bioleaching to supplement this process therefore, seems promising [12]. The main objectives of the present research work was to investigate the extraction of aluminum from low grade black shale ore using organic acid metabolites from acidophilic heterotrophs like *Aspergillus niger* and *Penicillium notatum.*

*To whom all correspondence should be addressed.

Experimental

In this research work, all the reagents and chemicals were of analytical grade. All culture media were purchased from Oxoid, UK.Acid standards for citric acid, oxalic acid, malic acid and tartaric acids were attained from Sigma-Aldrich Co. (St Louis, MO) USA.

Black shale sample

Black shale ore sample was taken from the Pakistan Institute of Engineering and Applied Sciences (PIEAS), Islamabad, Pakistan. That was somewhat alkaline (pH 7.3) and partially soluble in water. Black shale ore sample having black color might be due to the existence of carbon, hydrocarbon or iron sulfide that was present in it. Sample was dried at 105 °C and ground to size of <74 µm by strainer. Atomic absorption spectrophotometer showed that the sample contained Al 8799, Fe 3761, Cu 39.1, Zn 31.9, Co 18.3 and Ni 8.3 (mg/kg).

Scanning Electron Microscopy-Electron Dispersive X-Ray Spectroscopic analysis (SEM-EDX)

Analysis for the indication of elements present on the surface was performed by SEM-EDX spectroscopy (Hitachi S-2380, Japan). Sample was dried in air blower and standardized on the aluminum stub. EDX (Electron Dispersive X-ray Spectroscopy) analysis to find out the elements present at the shell of the picky area of the ore sample was performed at the same time [13].

Chemical leaching of aluminum

Chemical leaching tests were conducted using analytical reagent grade citric, oxalic, malic and tartaric acid in concentration of 0.05% (w/v), 0.1% (w/v), 0.5% (w/v) and 1% (w/v) with 1% (w/v) of black shale (ore suspension). Test runs were performed in triplicate. Flasks were shaken for the period of 10 days, and the pH was monitored every 2nd day. For aluminum, the supernatants were collected at the end, filtered and analyzed in each sample by UV/VIS spectrophotometer (Hitachi, UV-200; Japan) [11].

Analysis of aluminum in bioleached solution

Bio recovery of aluminum was determined spectrophotometrically at an absorbance wavelength of 540 nm after forming red complex with Eriochrom Cyanine R. Eriochrom Cyanine R was used as chromogenic agent [14].

Fungal strains and growth conditions

The cultures of already purified and identified microbial strains of *Penicillium notatum* as well as *Aspergillus niger* was used in the present research work. These cultures were further multiplied to obtain pure culture in liquid medium that consisted of NH_4NO_3, 2.0 g/L; KH_2PO_4, 5.0 g/L; $(NH_4)_2SO_4$, 4.0 g/L; peptone, 2.0 g/L; $MgSO_4.7H_2O$, 0.2 g/L; trisodium citrate, 2.5 g/L; and yeast extract, 1.0 g/L. The final volume was made up to 1000 mL with distilled water [14]. For bioleaching experiments, nine sets of 250-mL Erlenmeyer flasks (for each microbe) each containing 100 mL of culture medium, were prepared in triplicate. In each flask, medium was sterilized before the addition of 5.0 % (m/v) of given substrate after pretreatment then inoculated with 1.0 mL of *P. notatum* spore suspension (1.8×10^6spores mL^{-1}). *A. niger* spore suspension as inoculum (2.4×10^8 spores mL^{-1}) was added to another set of flasks. After sealing with removable cotton, all the flasks were incubated in an orbital shaker (Gellen Kamp, England) at 28°C with optimum velocity of agitation 150 rpm for the period of 15 days.

Pretreatments of substrates

The substrates were subjected to a pretreatment process before the addition into medium. Glucose that was used as substrate for medium 1, require no pretreatment. Cane molasses were diluted up to 50 % (v/v) before adding in to medium 2 as substrate. Other substrates like mango peel, seed cake and rice bran, were first oven dried, finely ground, weighed and then added as substrate in media 3, 5 and 7 (*A. niger* & *P. notatum*), respectively. For media 4, 6 and 8 (*A. niger* & *P. notatum*), Mango peel, seed cake and rice bran substrates were subjected to soaking treatment in sulphuric acid (pH 2) for 24 h, then weighed and added in the respective medium as illustrated in table 1. The control medium contained no any substrate under the same conditions.

Table-1: Pretreatments of substrates for *A. niger* (a) and *P. notatum* (p).

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

Leaching of the shale residue with organic acids produced by A. niger & P. notatum

Supernatants containing organic acid metabolites were used to leach the metals from the shale residue. An amount of 1.00% (w/v) of the ore suspension was added to each medium in the various flasks and incubated on an orbital shaker at 28 ºC with agitation speed of 150 rpm for the period of 36 days. Sampling was done at determined time intervals to register the changes in pH value, and dissolved aluminum concentration. Finally, the solid phase was separated, and the pH value of the liquor was measured by a Metrohm pH meter (model 744). The dissolved aluminum concentration was measured spectrophotometrically by the Eriochrom Cyanine R method [14].

Analysis of organic acids

For the qualitative and quantitative analysis of microbial metabolites present in supernatant liquors of the microbial cultures collected after 15 days of cellular growth was performed by HPLC. Before HPLC, supernatants were sterilized, centrifuged (8000 rpm for 10 min at 15ºC) and then filtered to remove any microbial cells [15]. Samples were vortexed after centrifugation and filtration. Filtered and sonicated acetic acid (0.25 % (v/v) was used as mobile phase. 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 into an analytical Hypersil (Thermo Hypersil, GmbH, Germany) ODS reverse phase (C_{18}) column (250×4.6 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. Detections were performed at a wavelength of 254 nm. Organic acids were identified by comparing the retention times and quantified on the basis of peak area 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).

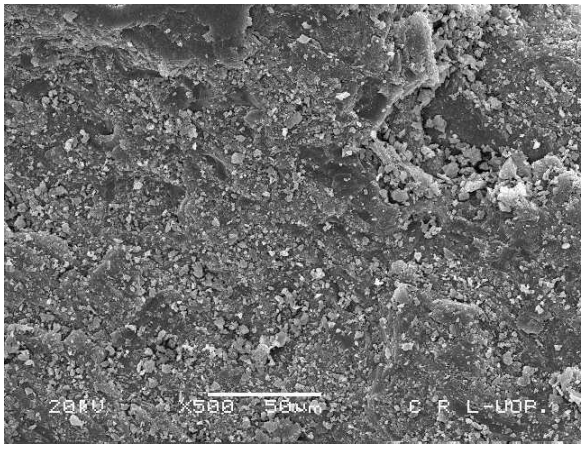
Statistical analysis

All the data of samples before and after bioleaching in triplicates were reported as mean ± SD [16].

Results and Discussion

Scanning Electron Microscopic-Electron Dispersive X-Ray Spectroscopy (SEM-EDX)

Scanning Electron Microscopy was carried out to observe the surface morphology of black shale ore sample. Electron dispersive spectroscopy (EDX) was simultaneously performed to detect the elements present on the surface of a particular area of the ore. The micrographs of the representative black shale samples (before and after leaching) with SEM analysis show that black shale is a aluminosilicate ore, comprised to a large extent of mica and siliceous materials of predominantly glassy and somewhat crystalline structure, with biogenic as well as chemical minerals. The biogenic minerals are (NiFe)xS, $(NiFe)S_2$, SiO_2 and Ca-phosphates as shown in Fig. 1a. In two of the selected bioleached samples of ore residue (for both microbes), sufficient erosion was observed on the surface and cracks and pits also developed, due to acidic leaching, as shown in Figs. 1b and 1c.



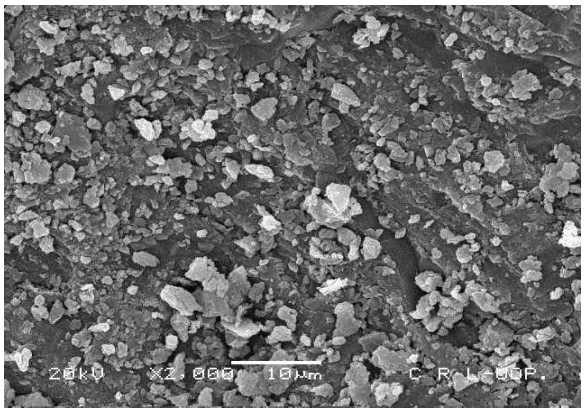
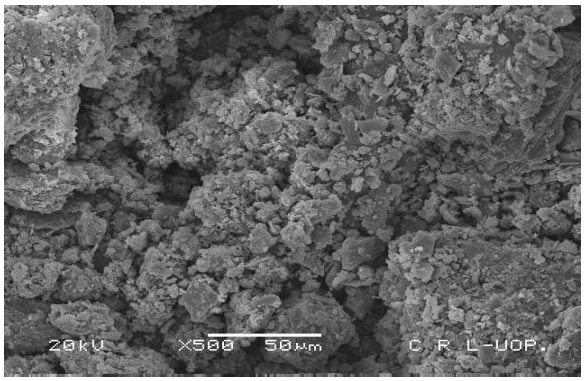


Fig. 1a: SEM micrograph of raw sample before bioleaching (a 50µm and b 10 µm)



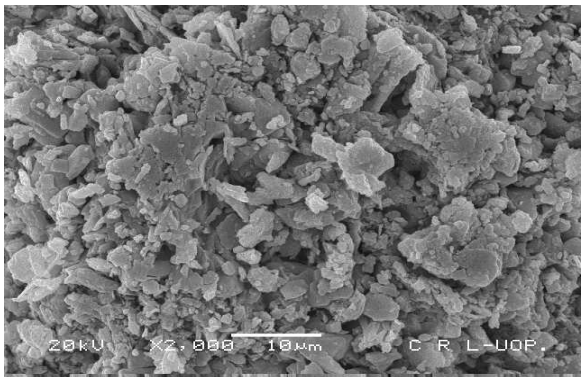
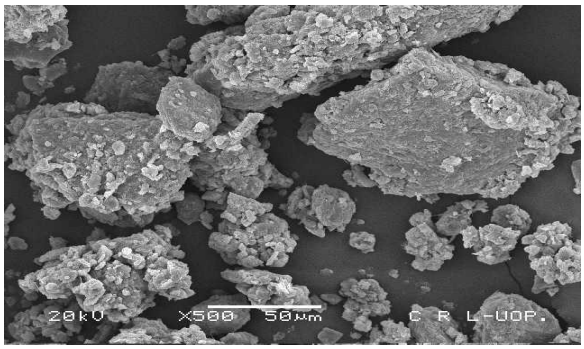


Fig.1b: SEM micrograph of bioleached sample by *A. niger* (a 50µm and b 10 µm).



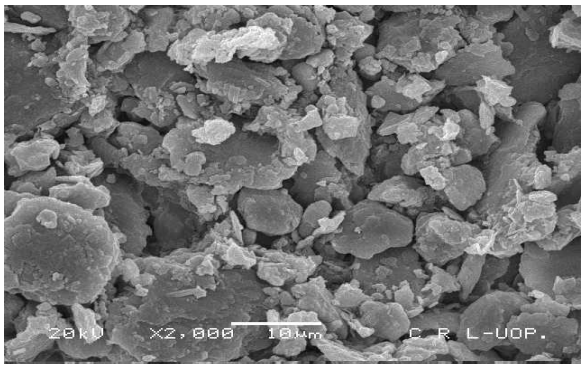


Fig.1c: SEM micrograph of bioleached sample by *P. notatum* (a 50µm and b 10 µm).

Similar results have been reported by Willscher and Bosecker [17] who concluded that erosion of siliceous particles was due to complexation of organic acids with metals. Elemental analysis of the exposed surface of the raw sample under Electron Dispersive X-Ray Spectroscopy (EDX) has shown in Fig. 2a. Major elements present on the exposed surface identified by EDX study were Fe, Si, Al, Ti, Cu, S, P, Cl, K, Na, Ca, C and O. After bioleaching, the apparent morphology was quite different, as shown in Figures 2b and 2c. In the bioleached samples of both strains, Al, Si and Fe were detected on the surface. Similar morphological characters of black shale have also been reported by Ghorbani *et al.*,[11]. Comparable findings have been reported by Anjum *et al.*,[15], while Bross *et al.*,[18] detected V, Ni, Co, Zn and Cu by EDX as major elements in black shale. Orberger *et al.*,[19] reported the presence of Cu, Ag, As, Mo, Se, Tl, Cd, platinum group elements and gold on the surface of black shale. The mineralogy of black shale changes according to the metamorphic grades of the sediments [20]. In black shale deposits have considerable amount of very rare, precious and heavy metals, these black shale deposits are distributed worldwide in these deposits the metals that are present are Se, As, Mo, Re, Pt, Cd, Sb [21-23], as well as P, Mn, Fe, Cu, Ni, Zn, Pb, Ag, Zn, Co, V, Sn, Bi, Au, U, Pd, Ti, and Si [24]. Metalliferous black shale are organic-rich rocks that are normally deepen in metallic elements like Mo, Ni, Si, P, S, Al, Fe, Co, Cu, Pb, U, Mn and As localized in specific horizons [25-26].

Chemical leaching

Chemical leaching tests were conducted using analytical reagent grade organic acids and conditions were optimized for the extraction of aluminum from the ore with malic (1%), citric (0.1%), oxalic (0.05%) and tartaric acid (0.5%), with a sample concentration of 1%. Increased aluminum concentrations were detected as the concentration of the acids and the pH of the medium increased. The aluminum dissolution was the highest (5379mgL^{-1}) in the shaking flask containing oxalic acid, followed by the shaking flask containing citric (4521 mgL^{-1}), malic (3410 mgL^{-1}) and tartaric acid (2695 mgL^{-1}) (Fig.3). under optimal condition, citric and oxalic acids have bear out to be the most competent leaching agents for aluminum dissolution. However, Santhiya and Ting [27] found oxalic acid the best medium to leach aluminum while Murad *et al.*,[28] reported significant recoveries of copper, nickel and cobalt with citric and oxalic acids.

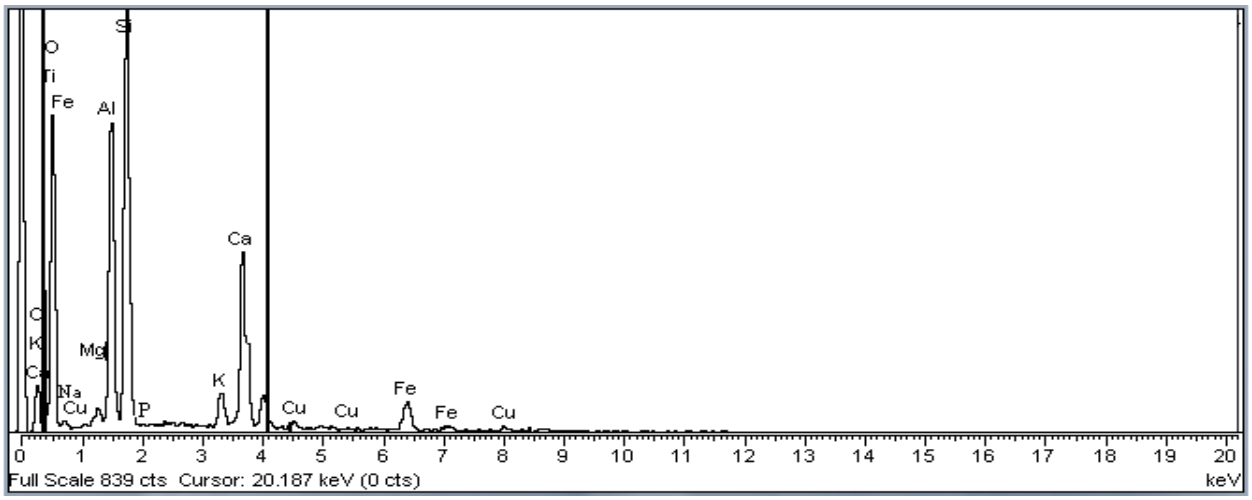


Fig. 2a: EDX analysis of the raw sample.

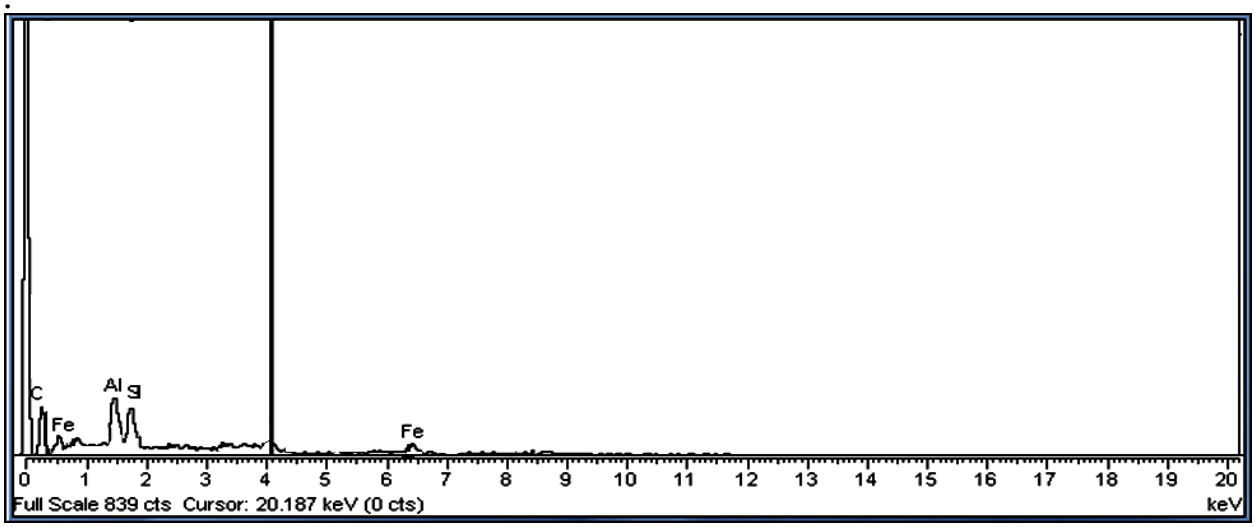


Fig. 2b. EDX analysis of the bioleached sample by *A. niger*

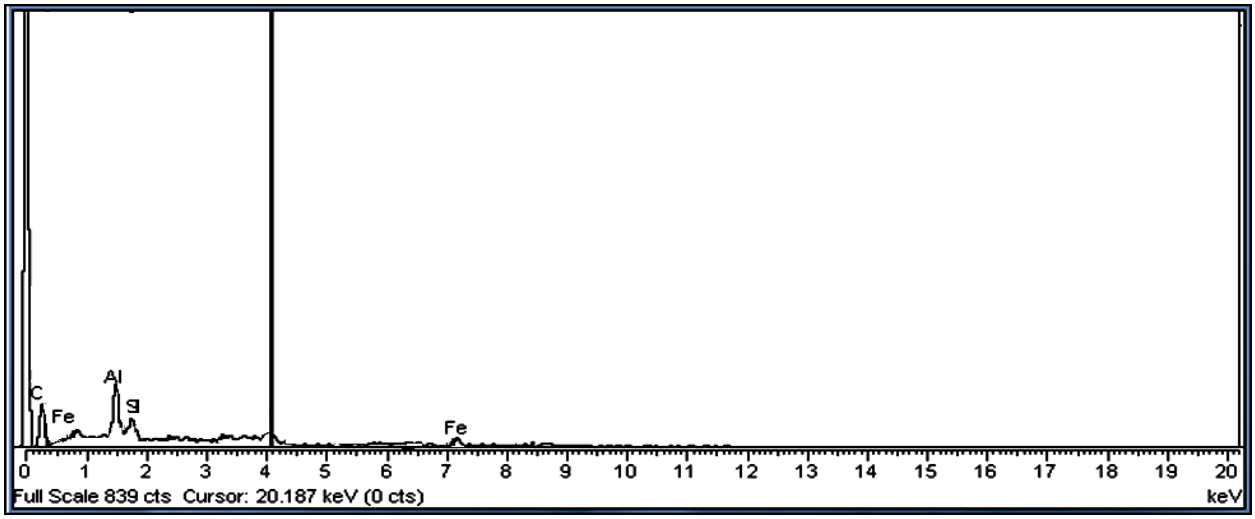


Fig. 2c: EDX analysis of the bioleached sample by *P. notatum.*

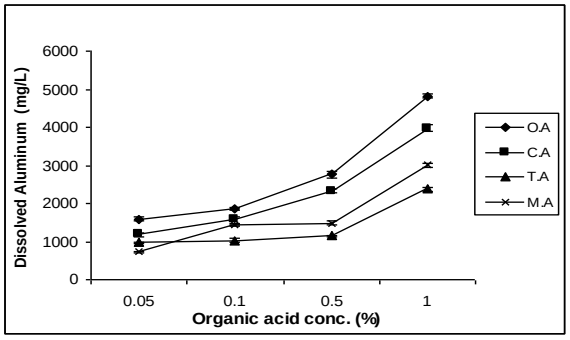


Fig. 3: Chemical leaching of aluminum by organic acids.

Morphological changes and biomass production

During the growth period of *P. notatum* minute bead like structures of yellow hue were produced. The volume of the beads increased with increased the time. Hyphae of *P. notatum* were branched with knob ends. During the end of growth periods small hyphae appeared and color change from light yellow to bluish green. The color of hyphae of *P. notatum* was colorless the hue that was appeared is due to their conidia. Contrary to this, in *A. niger* culture formed dark colored beads of different sizes in the inoculated flasks. The same observations have also been made earlier by Murad *et al.*,[28] and Anjum *et al.*,[29]. A considerable decline in pH was observed in each case of the growth medium at the end of growth period, excluding the control flask which contained no substrate. In *A. niger*, the maximum biomass production (6.99 %) was observed in the medium having the glucose substrate as standard, then in the molasses substrate (6.56%), and the minimum biomass production (0.32 %) occurred in the medium having rice bran substrate (Fig. 4). Aung and Ting [30] and Santhiya and Ting [27] reported biomass yields of 2.58% up to 15 days of growth of the same microbe.

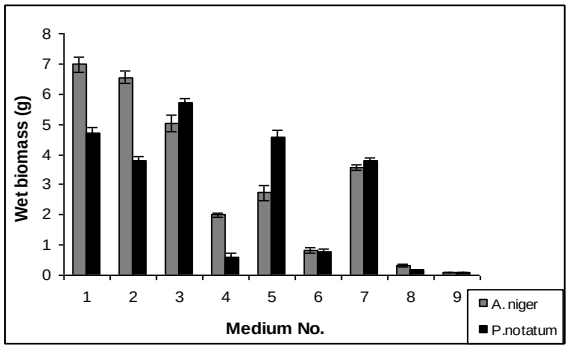


Fig. 4: Wet biomass production by *A. niger* and *P. notatum.*

In acidified mango peel medium the *P. notatum*, showed the maximum biomass yield (5.71 %), while the least biomass (0.17%) was found in the rice bran medium (Fig. 4). High yields of biomass indicate more utilization of the substrate, whereas the low yield of biomass production in the rice bran medium might be due to the presence of more cellulosic material and less available free glucose for the microbes. A maximum biomass (wet) production is harmonizing to acid production [15]. Substrates oxidation by microorganism produces acid. During growth studies of the heterotrophs, the substrate is oxidized by microbes and organic acids like malic, oxalic, citric and tartaric acid which plays an elemental role in the extraction of metals [31]. The acids that are produced also play a role in complexing agents with two or more electron donors for metals. Tartaric, citric and oxalic acid have chelating properties and large affinities for metals [15, 31].

Organic acid analysis

To confirm that *A. niger* as well as *P. notatum* was indeed producing organic acids, culture medium was analyzed by HPLC 15 days after inoculation and samples collected on the 36th day of leaching. The HPLC data of the organic acids produced by *Aspergillus niger* are shown in Table 2a whereas data of organic acid metabolites produced by *Penicillium notatum* shown in table 2b is already reported by Anjum *et al.*,[29]. The results are clearly demonstrating the capabilities of microbes for acid production. HPLC data clear that *A. niger* is more efficient in producing organic acids than *P. notatum.* After 15 days of incubation of each analyzed sample it was clear that in both microbes, citric acid was the main acid product followed by malic, oxalic and tartaric acid. In both cases, the pH fell to its minimum value during the 15 days of growth period [29]. The concentration of organic acids rose higher in those medium having acidic pretreatment of substrates than in cases where different conditions exist.

In *Penicillium notatum*, the maximum citric acid (1.11%) was also detected in the acidified mango peel medium, but the concentration was lower than that produced by *A. niger*. Malic (1.32%) and oxalic acid (0.43%) were found in higher concentrations in the molasses medium than in the case of *A. niger*. On the other hand, oxalic acid was just formed in media 1, 2, 4 and 8 in minor concentration (0.01-0.43 %). The recorded pH dropped to the level of 3.0-5.4. Tartaric acid was only found in media 2, 4 and 6 (0.04-0.10%). These agricultural wastes have maximum quantity of structural fiber; can therefore be used as substrates by microbes as an essential requirement for continuance, development, reproduction and production [32]. *A. niger* is more efficient in exploiting organic wastes as a substrate by its enzymatic activity than *P. notatum.* Moreover, acidic conditions favor and enhance its activity to a greater degree than in *P. notatum.*

Table-2a: Concentration of organic acids in fermented media after 15 days of growth of *A. niger* (before leaching) as well as after 36 days of leaching.

Medium No.		Organic acids (%) w/v)				
		pH of fermented media	Citric acid	Malic acid	Oxalic acid	Tartaric acid
1	Before leaching	3.7	1.17 ± 0.03	0.99 ± 0.07	0.78 ± 0.05	0.04 ± 0.001
	After leaching	8.1	0.09 ± 0.005	ND	ND	ND
2	Before leaching	3.6	1.51 ± 0.07	0.91 ± 0.04	0.51 ± 0.05	ND
	After leaching	7.9	0.07 ± 0.001	< 0.01	ND	ND
3	Before leaching	3.5	0.03 ± 0.00	ND	ND	< 0.01
	After leaching	7.7	ND	ND	ND	ND
4	Before leaching	3.7	1.79 ± 0.09	1.01 ± 0.05	0.22 ± 0.04	0.14 ± 0.03
	After leaching	8.2	0.04 ± 0.00	ND	ND	ND
5	Before leaching	4.8	0.06 ± 0.00	ND	ND	ND
	After leaching	7.8	ND	ND	ND	ND
6	Before leaching	4.1	1.23 ± 0.02	0.72 ± 0.06	0.43 ± 0.06	0.07 ± 0.00
	After leaching	8.1	0.03 ± 0.00	ND	ND	ND
7	Before leaching	4.4	0.02 ± 0.00	ND	ND	ND
	After leaching	7.2	ND	ND	ND	ND
8	Before leaching	3.9	1.01 ± 0.05	0.33 ± 0.01	< 0.01	< 0.01
	After leaching	8.0	< 0.01	ND	ND	ND

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

Table-2b: Concentration of organic acids in fermented media after 15 days of growth of *P. notatum* (before leaching) as well as after 36 days of leaching.

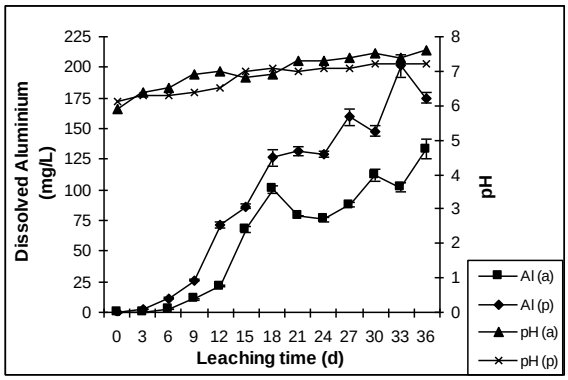
Medium No.		Organic acids (%) w/v)				
		pH of fermented media	Citric acid	Malic acid	Oxalic acid	Tartaric acid
1	Before leaching	3.2	0.69 ± 0.035	0.99 ± 0.05	0.01± 0.001	ND
	After leaching	7.9	ND	< 0.01	ND	ND
2	Before leaching	5.4	0.47 ± 0.025	1.32 ± 0.03	0.43 ± 0.02	0.05 ± 0.001
	After leaching	8.1	0.02 ± 0.00	ND	ND	ND
3	Before leaching	4.0	0.12 ± 0.005	0.07 ± 0.004	ND	ND
	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
	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
	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
	After leaching	7.9	ND	< 0.01	ND	ND
7	Before leaching	4.7	0.06 ± 0.00	< 0.01	ND	ND
	After leaching	7.9	ND	ND	ND	ND
8	Before leaching	3.0	0.52 ± 0.01	0.73 ± 0.03	0.04 ± 0.001	ND
	After leaching	7.2	< 0.01	< 0.01	ND	ND

(Anjum et al., 2009)

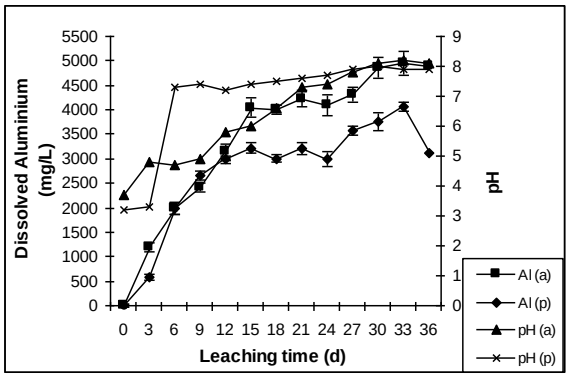
Aspergillus niger produced high concentrations of citric and malic acid (1.79 & 1.01%) in an acidified mango peel medium with a pH of 3.70. This might be due to the partial degradation of the cellulosic structure by acid treatment which releases more free carbon for the microbes to utilize during the citric acid production process. Other media also contained significant but comparatively lower concentrations of citric acid than that of the acidified mango peel medium. Oxalic acid was detected only in media 1, 2, 4 and 6 (0.22-0.78%). Significant amounts of citric and oxalic acid production under similar conditions have also been reported by Aghaie *et al.*,[33] and Li *et al.*,[34]. Tartaric acid was only detected in media 1, 4 and 6 in small amount (0.04-0.14%). These acids are eminent lixiviants for the leaching of metals from ore materials [35] and commercially coat effective for leaching of heavy metal and eco friendly. Similar findings were reported by Santhiya and Ting [27]. Thangavelu *et al.*,[36] found that complexing agents like malate, citrate and lactate are also produced from fungi that have significant role in bioleaching of metals from their ores.

Biorecovery of aluminum

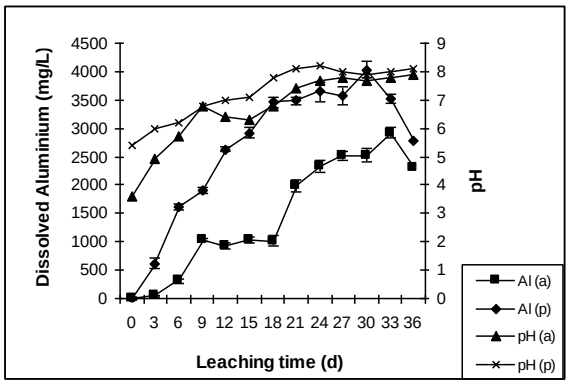
Supernatants of all media containing organic acid metabolites were filtered and collected. Then the ore slurry (1 % w/v) was added to determine the aluminum extraction. In the control medium of *A. niger*, as well as *P. notatum*, containing no substrate, an increasing trend in pH was noted during the growth period of 15 days. At the end, the pH value approached 5.9 and 6.1 respectively. Because there was decline in carbon which was source of microorganism so there will be production of no complexing agents. The same trend has been reported previously by Anjum *et al.*,[15]. After addition of ore, a significant increase in pH was recorded this might be due to dissolution of minerals from their ore in medium. At the end of the leaching period the pH was 7.6 and 7.2, with little recovery of aluminum of 151 mgL^{-1} and 229 mgL^{-1} respectively (Fig. 5a).


Dissolved Aluminium (mg/L)
pH
Leaching time (d)
Al (a)
Al (p)
pH (a)
pH (p)

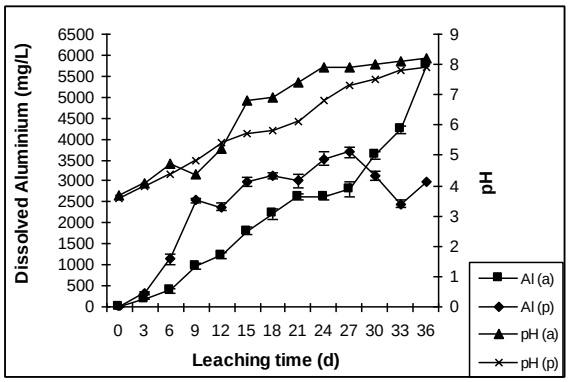
(a)


Dissolved Aluminium (mg/L)
pH
Leaching time (d)
Al (a)
Al (p)
pH (a)
pH (p)

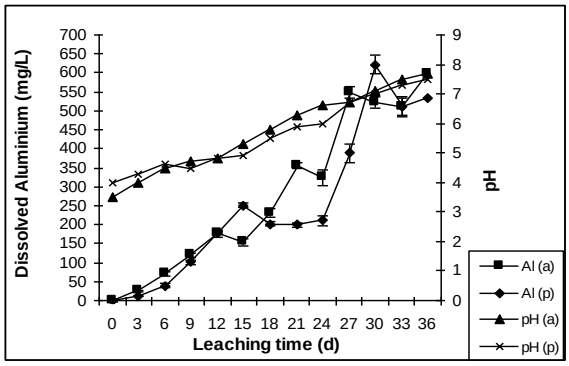
(b)


Dissolved Aluminium (mg/L)
pH
Leaching time (d)
Al (a)
Al (p)
pH (a)
pH (p)

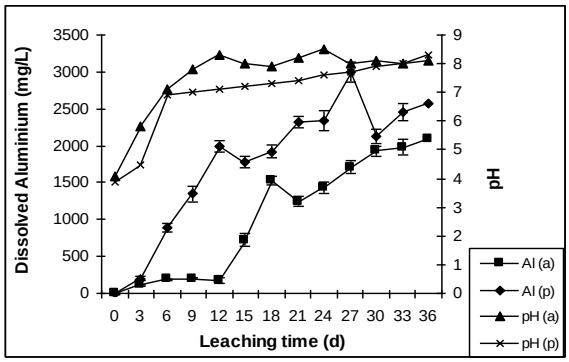
(c)


Dissolved Aluminium (mg/L)
pH
Leaching time (d)
Al (a)
Al (p)
pH (a)
pH (p)

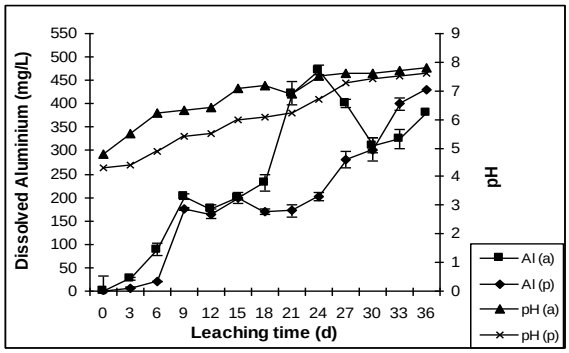
(d)


Dissolved Aluminium (mg/L)
pH
Leaching time (d)
Al (a)
Al (p)
pH (a)
pH (p)

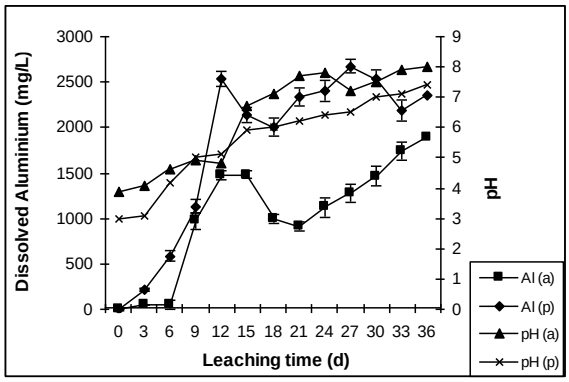
(e)


Dissolved Aluminium (mg/L)
pH
Leaching time (d)
Al (a)
Al (p)
pH (a)
pH (p)

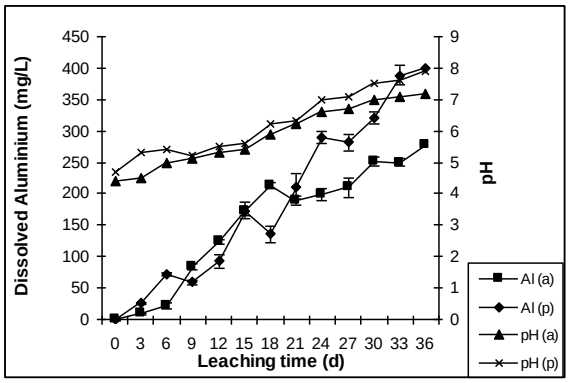
(f)


Dissolved Aluminium (mg/L)
pH
Leaching time (d)
Al (a)
Al (p)
pH (a)
pH (p)

(g)


Dissolved Aluminium (mg/L)
pH
Leaching time (d)
Al (a)
Al (p)
pH (a)
pH (p)

(h)



(i)

Fig. 5: 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)

In the *A. niger* media containing different substrates, a decrease in pH was noted (3.6-4.8) during the growth period of 15 days, which is due to production of acids by incomplete oxidation of glucose. After 15 days of growth, bioleaching started. As bioleaching progressed, the pH progressively increased to a maximum of 8.3, which indicates the consumption of organic acids due to aluminum dissolution. The maximum amount of aluminum (6571 mgL^{-1}) was detected in the acidified mango peel medium (Fig. 5e) that contained the maximum concentration of citric acid. It was followed by the medium with glucose (5559 mgL^{-1}), molasses (3320 mgL^{-1}), acidified seed cake (2376 mgL^{-1}) and acidified rice bran (2148 mgL^{-1}), as shown in Figures 5b, 5c, 5g and 5i respectively. In these media, the maximum recovery of Al can be correlated with the organic acid production from the metabolic activities of the fungus [37]. However, it also involves the oxalic and malic acids and this observation is corroborated by earlier reported studies of Ghorbani *et al.*,[38] and Xu and Ting [39].

Media 3, 5 and 7 that were not treated by acid (Fig. 5d, 5f and 5h), contained only a small amount of aluminum, which might be due to the presence of a small amount of citric as well as other acids that solubilize the aluminum. The same trend for aluminum was found in *P. notatum*. The maximum recovery of aluminum was detected in the glucose containing medium (4627 mgL^{-1}), as well as molasses containing medium (4582 mgL^{-1}), as can be observed from the data in Fig. 5b and 5c. As the pH decreased during growth period, acid production was increased. Other acidified media, like mango peel (4184 mgL^{-1}), seed cake (3411 mgL^{-1}) and rice bran (3038 mgL^{-1}) also showed significant dissolution of aluminum, as is clear from the data displayed in Fig. 5e, 5g and 5i. Non acidified medium showed the same trend of metal recovery as in the *A. niger* Case.

For the two strains employed, aluminum dissolution increased with time and reached its maximum value after 36 days of leaching. Different environmental adaptations or a difference in the optical properties produced a variation in alumina dissolution by both microbes. The fungi are able to leach metals by acidolysis and complex formation due to the presence of different organic acids in the medium which stimulate the disbanding of metals [39-40]. As production of organic acids provide anions and protons for metal leaching by acidolysis and complexation [41]. In acidolysis, the metal is solublized through organic or inorganic acid produced by the microorganism. In complexation, the anions form complexes with the metal cations to produce metal complexes. Low pH values (>7) favors acidolysis, with the release and enhanced mobility of free metal cations by protonation [36].

Citric acid is a tricarboxylic acid and contains three carboxylic groups and one hydroxyl group as a possible proton donor at 28°C. When Al^{+3} present in the system and citric acid is fully dissociated in the aqueous solution, the following complexation reaction may take place [11].

$C_6H_8O_7 \rightarrow (C_6H_5O_7)^{-3} + 3H^+$ ($pK_a3=6.39$)
$(C_6H_5O_7)^{-3} + Al^{+3} \rightarrow Al(C_6H_5O_7)$

Similarly, oxalic acid contains two carboxylic groups ($pK_a1= 1.20$ and $pK_a2= 4.20$) and form aluminum oxalate [11]. Insoluble aluminum oxalate has been precipitated, but could not be detected by X-ray diffraction because of its amorphous nature. The results of a scanning electron micrograph (SEM) examination of the residue after the attack by organic acid metabolites did not show spores or vegetative filaments of the fungus (Fig. 1b & 1c). In addition to organic acid production, this fact suggests an indirect mechanism of attack on the mineral by *A. niger* as well as *P. notatum*, which can be represented by the following reaction scheme

$C_2H_2O_4 \rightarrow (C_2HO_4)^- + H^+$ ($pK_{a1}=1.20$)
$3(C_2HO_4)^- + Al^{+3} \rightarrow Al(C_2HO_4)_3$

Mass balances

At the end, a mass balance was performed for aluminum by residual solids. The amount of aluminum was measured in residue and then added in

amount that was produced or dissolved in supernatants, and compared with initial amount of aluminum in ore. It was found that a quantity of the aluminum (2035 mgL^{-1}) has not been accounted for. This amount of aluminum might be lost in any of the process like sample preparation or in process of filtration, in filtration the salts might be absorbed on filter paper surface.

Conclusion

This study describes the recovery of aluminum from black shale by organic acids that have been generated by *A. niger* and *P. notatum*. Different agricultural wastes were utilized as substrates to produce organic acids. Organic acid production was improved by the adding up sulphuric acid (H_2SO_4) to hydrolyze cellulose and lignin materials. Aluminum dissolution obtained with *A. niger* was better than *P. notatum*. The maximum recovery of aluminum (6571 mgL^{-1}) was found in an *A. niger* medium containing acidified mango peel substrate at 28ºC after 36d of leaching at 150 rpm. Microbiological activity, apart from bio-acid production contributed to the metals extraction process.

Acknowledgements

The authors are highly thankful for the kind support from plant pathology laboratory of Department of Plant Pathology for the microbial work and the Institute of Soil Sciences at the University of Agriculture, Faisalabad for elemental analysis.

References

1. D. E. Rawlings, Heavy Metal Mining Using Microbes, *Annual Reviews in Microbiology*, **56**, 65 (2002).
2. F. Baldi, V. Leonardi, A. D'Annibale, A. Piccolo, F. Zecchini, and M. Petruccioli, Integrated Approach of Metal Removal and Bioprecipitation Followed by Fungal Degradation of Organic Pollutants from Contaminated Soils, *European Journal of Soil Biology*, **43**, 380 (2007).
3. A. Mishra, N. Pradhan, R. Kar, L. Sukla, and B. Mishra, Microbial Recovery of Uranium Using Native Fungal Strains, *Hydrometallurgy*, **95**, 175 (2009).
4. S. Brochot, M. Durance, J. Villeneuve, P. d'Hugues, and M. Mugabi, Modelling of the Bioleaching of Sulphide Ores: Application for the Simulation of the Bioleaching/Gravity Section of the Kasese Cobalt Company Ltd Process Plant, *Minerals engineering*, **17**, 253 (2004).
5. F. Anjum, H. N. Bhatti, and M. A. Ghauri, Enhanced Bioleaching of Metals from Black Shale Using Ultrasonics, *Hydrometallurgy*, **100**, 122 (2010).
6. S. K. Jain, A. A. Khan, and M. K. Rai, *Geomicrobiology*. CRC Press, Boca Raton, Florida, 61, 1 (2016)
7. Y. Zhou, X. Huang, G. Huang, X. Bai and Y. Li. Cu and Fe Bioleaching in Low-Grade Chalcopyrite and Bioleaching Mechanisms using *Penicillium janthinellum* Strain GXCR. *Chinese. J. Biotechnol*., **24**, 1993 (2008).
8. J. Zhang, A. X. Wu, Y. M. Wang and X. S. Chen. Experimental Research in Leaching of Copper-Bearing Tailings Enhanced by Ultrasonic Treatment (2008).
9. Z. Zhao, G. Zhang, G. Huo and H. Li, Kinetics of Atmospheric Leaching Molybdenum from Metalliferous Black Shales by Air Oxidation in Alkali Solution, *Hydrometallurgy*, **97**, 233 (2009).
10. R. McDonald and B. Whittington, Atmospheric Acid Leaching of Nickel Laterites Review: Part I. Sulphuric Acid Technologies, *Hydrometallurgy*, **91**, 35 (2008).
11. Y. Ghorbani, M. Oliazadeh, and A. Shahvedi, Aluminum Solubilization from Red Mud by Some Indigenous Fungi in Iran, *J Applied Biosci.*, **7**, 207 (2008).
12. E. Aghaie, M. Pazouki, M. Hosseini, M. Ranjbar, and F. Ghavipanjeh, Response Surface Methodology (RSM) Analysis of Organic Acid Production for Kaolin Beneficiation by *Aspergillus niger*, *Chem Eng J.*, **147**, 245 (2009).
13. A. Szubert, M. Łupiński, and Z. Sadowski, Application of Shrinking Core Model to Bioleaching of Black Shale Particles, *Physicochem ProbMine Proc.*, **40**, 211 (2006).
14. F. Anjum. H. N. Bhatti, M. A. Ghauri. Enhanced Bioleaching of Metals from Black Shale Using Ultrasonics. *Hydrometallurgy*, 3, 122 (2010).
15. F. Anjum, H. N. Bhatti, M. Asgher, and M. Shahid, Leaching of Metal Ions from Black Shale by Organic Acids Produced by *Aspergillus niger*, *Applied Clay Science*, **47**, 356 (2010).
16. R. G. Steel, J. H. Torrie, and D. A. Dickey, *Principles and Procedures of Statistics: A biological approach*. 1997: McGraw-Hill.
17. K. Bosecker, Bioleaching: Metal Solubilization by Microorganisms, *FEMS Microbiology reviews*, **20**, 591 (1997).
18. E. Brosse, Mineral Geochemistry Of Black

Shales From The Mid-Cretaceous Of The North-Atlantic, Dsdp Site-386 And Site-391, *Revue De L Institut Francais Du Petrole*, **38**, 299 (1983).

19. B. Orberger, J. Pasava, J. P. Gallien, L. Daudin, and P. Trocellier, Se, As, Mo, Ag, Cd, In, Sb, Pt, Au, Tl, Re Traces in Biogenic and Abiogenic Sulfides from Black Shales (Selwyn Basin, Yukon territories, Canada): a Nuclear Microprobe Study, *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms*, **210**, 441 (2003).
20. S. Agnieszka, M. Lupinski and Z. Sadowski. Application of Shrinking Core Model to Bioleaching of Black Shale Particles. *Fizykochemiczne Problemy Mineralurgii*, **40**, 211 (2006).
21. Z. Bao. Noble Metal Mineralization in the Black Shale of Northwestern Hunan. *Kuangwu Yanshi*, **17**, 70 (1997).
22. R. A. Berner, Atmospheric Oxygen Over Phanerozoic Time, *Proceedings of the National Academy of Sciences*, **96**, 10955 (1999).
23. R. Berner, S. Petsch, J. Lake, D. Beerling, B. Popp, R. Lane, E. Laws, M. Westley, N. Cassar, and F. Woodward, Isotope Fractionation and Atmospheric Oxygen: Implications for Phanerozoic O 2 evolution, *Science*, **287**, 1630 (2000).
24. B. Yu, H. Dong, E. Widom, J. Chen, and C. Lin, Geochemistry of Basal Cambrian Black Shales and Cherts from the Northern Tarim Basin, Northwest China: Implications for Depositional Setting and Tectonic History, *J Asian Earth Sci.*, **34**, 418 (2009).
25. E. Burford, M. Fomina, and G. Gadd, Fungal Involvement in Bioweathering and Biotransformation of Rocks and Minerals, *Mineralogical Magazine*, **67**, 1127 (2003).
26. M. Carolina, L. Racatala, M. Paris and J. Sanchez. A Comparison of Two Digestion Methods for the Analysis of Heavy Metals by Flame Atomic Absorption Spectroscopy.**19**, 23 (2007).
27. D. Santhiya and Y. P. Ting, Bioleaching of Spent Refinery Processing Catalyst Using *Aspergillus niger* with High-Yield Oxalic Acid, *J Biotechnol.*, **116**, 171 (2005).
28. M. A. El-Holi and S. Al-Delaimy, Citric Acid Production from Whey with Sugars and Additives by *Aspergillus niger, AfrJ Biotechnol.*, **2**, 356 (2003).
29. F. Anjum, H. N. Bhatti, M. A. Ghauri, I. A. Bhatti, M. Asgher, and M. R. Asi, Bioleaching of Copper, Cobalt and Zinc from Black Shale by *Penicillium notatum, AfrJ Biotechnol.,* **8**, 19 (2009).
30. K. M. M. Aung and Y. P. Ting, Bioleaching of Spent Fluid Catalytic Cracking Catalyst Using *Aspergillus niger*, *Journal of Biotechnology*, **116**, 159 (2005).
31. X. Hai, Y. Daoyong, and Q. Guohe, Characterization of Petroporphyrins in Gudao Residue by UV–Vis Spectrophotometry Laser Desorption Ionization-Time of Flight Mass Spectrometery, *Fuel*, **84**, 647 (2005).
32. H. Ikram-ul, S. Ali, M. Qadeer, and J. Iqbal, Citric Acid Production by Selected Mutants of *Aspergillus niger* from Cane Molasses, *Bioresource Technology*, **93**, 125 (2004).
33. E. Aghaiea, M. Pazoukib, M. R. Hosseinia, M. Ranjbara and F. Ghavipanjeh. Response Surface Methodology (RSM) Analysis of Organic Acid Production for Kaolin Beneficiation by *Aspergillus niger* Chem Eng J., **147**, 245 (2009).
34. L. Le, J. Tang, D. Ryan, and M. Valix, Bioleaching Nickel Laterite Ores Using Multi-Metal Tolerant *Aspergillus foetidus* Organism, *Minerals Engineering*, **19**, 1259 (2006).
35. S. Heuberger, *Kinematics of the Karakoram-Kohistan suture zone, Chitral, NW Pakistan*. 2004.
36. V. Thangavelu, J. Tang, D. Ryan, and M. Valix, Effect of Saline Stress on Fungi Metabolism and Biological Leaching of Weathered Saprolite Ores, *Minerals engineering*, **19**, 1266 (2006).
37. J. Farbiszewska-Kiczma, T. Farbiszewska, and M. Bąk, Bioleaching of Metals from Polish Black Shale in Neutral Medium, *Physicochem Prob Min Proc.*, **38**, 273 (2004).
38. Y. Ghorbani., I. M. Oliazadeh and A. Shahvedi. Aluminum Solubilization from Red Mud by Some Indigenous Fungi in Iran. *Journal of Applied Biosciences*, (7), 207 – 213 (2008).
39. T. J. Xu and Y. P. Ting, Optimisation on Bioleaching of Incinerator Fly Ash by *Aspergillus niger*–Use of Central Composite Design, *Enzyme and Microbial Technology*, **35**, 444 (2004).
40. J. U. Lee, S. M. Kim, K. W. Kim, and I. S. Kim, Microbial Removal of Uranium in Uranium-Bearing Black Shale, *Chemosphere*, **59**, 147 (2005).
41. H. J. Lu and J. Wang, Ore Potential and Modes of Occurrence of Associated Elements in Lower Cambrian Black Shale of Western Zhejiang, *Mine Deposit*, **24**, 567 (2005