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CHARACTERIZATION OF SULFUR-OXIDIZING BACTERIA ISOLATED FROM ACID MINE DRAINAGE AND BLACK SHALE SAMPLES

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Abstract

Acid mine drainage (AMD) and black shale (BS) are the main habitats of sulfur-oxidizing bacteria. The aim of this study was to isolate and characterize sulfur-oxidizing bacteria from extreme acidic habitats (AMD and BS). Concentration of metals in samples from AMD and BS varied significantly from the reference samples and exceeded the acceptable limits set by the Environmental Protection Agency (EPA) and the World Health Organization (WHO). A total of 24 bacteria were isolated from these samples that were characterized both morphologically as well as through biochemical tests. All the bacteria were gram-negative rods that could efficiently oxidize sulfur into sulfate ions (SO_4^{-2}), resulted into decrease in pH up to 1.0 when grown in thiosulfate medium with initial pH 4.0. Out of 24, only 06 isolates were selected for phylogenetic analysis through 16S rRNA sequencing, on the basis of maximum sulfur-oxidizing efficiency. The isolates were identified as the species from different genera such as *Alcaligenes*, *Pseudomonas*, *Bordetella*, and *Stenotrophomonas* on the basis of maximum similarity index. The concentration of sulfate ions produced was estimated in the range of 179-272 mg/L. These acidophiles might have various potential applications such as biological leaching of metals from low-grade ores, alkali soil reclamation and to minimize the use of chemical S-fertilizers and minimize environmental pollution.

Key words: Sulfur-oxidizers, Acid mine drainage, Black shale, *Alcaligenes*, *Pseudomonas*, *Bordetella*, *Stenotrophomonas*.

Introduction

Acidophiles are extremophilic organisms (mostly microorganisms) that can survive and grow in environment of low pH from 0 to 4 (Johnson, 2012). They inhabit pristine environments like geothermal areas, volcanic, black shale and acid rock drainage (ARD) (Gonzalez *et al*., 2003). They also found in low pH environment of anthropogenic origin like acid mine drainage (AMD) and bioleaching heaps (Johnson & Hallberg, 2003). Acidophilic bacteria naturally inhabit in these environments assist in the breakdown of naturally present sulfide minerals like pyrite (FeS_2), arsenopyrite (FeAsS), chalcopyrite ($CuFeS_2$), (Cu_2S, CuS, CuS_2), Ni-sulfide (NiFe)S, $(NiFe)S_2$, sphelrite ((Zn,Fe)S), galena (PbS), and pyrrhotite (Fe_1-xS). These sulfides are oxidized in the presence of water and oxygen into sulfate ions that leads to acidic rich drainage (Akcil & Koldas, 2006). Mostly sulfur oxidizing bacteria (SOB) belong to *Thiobacillus*, *Thiomicrospira*, *Thiothrix*, *Desulfuromonas* and *Achromatium* genera. However, sulfur oxidation is not limited only to true sulfur bacteria but also other heterotrophic bacteria isolated from soil and marine environment carry the same process (Das *et al*., 1996). The heterotrophic bacteria belong to genera *Xanthobacter* (Cho *et al*., 1992), *Pseudomonas* (Sorokin *et al*., 1999), *Escherichia coli* strains (Starkey, 1935) involve in sulfur oxidation. *Acidithiobacillus thiooxidans* is a chemolithotrophic acidophilic bacterium that oxidizes both elemental sulfur and sulfides into sulfate, so it plays significant role in bioleaching of metals from these sulfide ores (Lundgren & Silver, 1980; Brierley, 1982) which results into production of higher concentration of sulfate ions in order to achieve higher bioleaching efficiency. However, *At. thiooxidans* and other sulfur oxidizing acidophiles have slow growth rate, therefore, low sulfate ion production (Kurosawa *et al*., 1991). The maximum sulfate concentration and cell density in conventional shaking flask cultivation was estimated as about 15000ppm (Liu *et al*., 2003) and 0.224g cell/L (Butler, 1975), respectively, within 8–11 days of incubation.

Water with high concentration of sulfates draining out from these sulfide ores sites and contaminate in land water supplies that has extremely harmful effects on aquatic fauna and flora, corrosion of bridge abutments, destruction culverts, roads and other important structures, also makes water resources so turbid as to be unfit for recreational activities (Bigham & Nordstrom, 2000). Mining processes of several minerals like gold, nickel and copper associate with problem of AMD that has bad effects on waterways and biodiversity. Also, effluents generated by some mining industry contain high amount of toxic substances like heavy metals and cyanides, which has serious effect on human health and ecology (Azapagic, 2004). Concentrations of elements like Zn, Cu, Al, Fe and Mn increase in water with low pH. AMD is not only a big source for groundwater and surface pollution, but also for the degradation of surrounding soil quality and disperse heavy metals into the environment (Hallberg, 2010).

Phylogenetic and functional description of microbial diversity in AMD and BS of Khala Chatta has not been explored yet. This study will help us in understanding the metabolic characteristics of microorganisms against sulfur and other minerals in extreme environments. Bioleaching of metals from their ores is directly proportional to sulfates formation by acidophilic microorganism. Various sulfur-oxidizing bacteria were isolated from AMD and BS in our study and sulfur oxidation efficiency of these bacteria was also investigated.

Materials and Methods

All chemicals and other reagents used in present study were of analytical grade and obtained from Merck and Sigma-Aldrich Chemical Co.

Sampling procedures: The liquid and soil samples were aseptically collected from acid mine drainage and black shale (Fig. 1) of Khala Chatta (2394m; 34°02.675'N 073˚24.201'E). Both surface and subsurface soils and water from inside and outside mines were collected in sterilized polythene zipper bags and polypropylene screw capped Nalgene bottles that were properly labelled. The pH and temperature of site was noted down with pH meter (Eutech Instruments, pH 1,500) and thermometer. Brief description of each sample is given in Table 1. The samples were stored at 4 ± 0.1°C in refrigerator for further analysis.

Samples analysis by atomic absorption: For the quantitative analysis of various metals in the samples, Atomic absorption Spectrophotometry (AA240FS Fast Sequential Atomic Absorption Spectrophotometer) was performed. Each sample was analyzed in triplicate and mean value of absorbance was used to calculate the heavy metals concentration. For this purpose the water samples were filtered and made them acidic with HNO_3, while for soil samples soil digestion procedure was performed. About 1 g of powdered soil was mixed with 15mL aqua regia (3:1, HNO_3:$HClO_4$), and heated at 150°C and was left overnight, 5 mL $HCLO_4$ was added and heated again; the solution became almost dry until brown fumes were off ramp. Whatman filter paper (No 42) was used for filtration and volume was raised to 50 mL with double distilled water. The metals concentration in samples was compared with that of ordinary garden soil from the lawn (non-cultivated) and ordinary stream water (flow continuously and strike with rocks from the hilly area) as control and with the permissible ranges of these metals from WHO (World Health Organization, Permissible limits of heavy metals in soil and plants, 1996, International year of fresh water, 2003 and Guidelines for drinking water quality, 2008) and EPA (Environmental Protection Agency USA, Risk Assessment Guidance for Superfund, Human Health Manual, 1985).

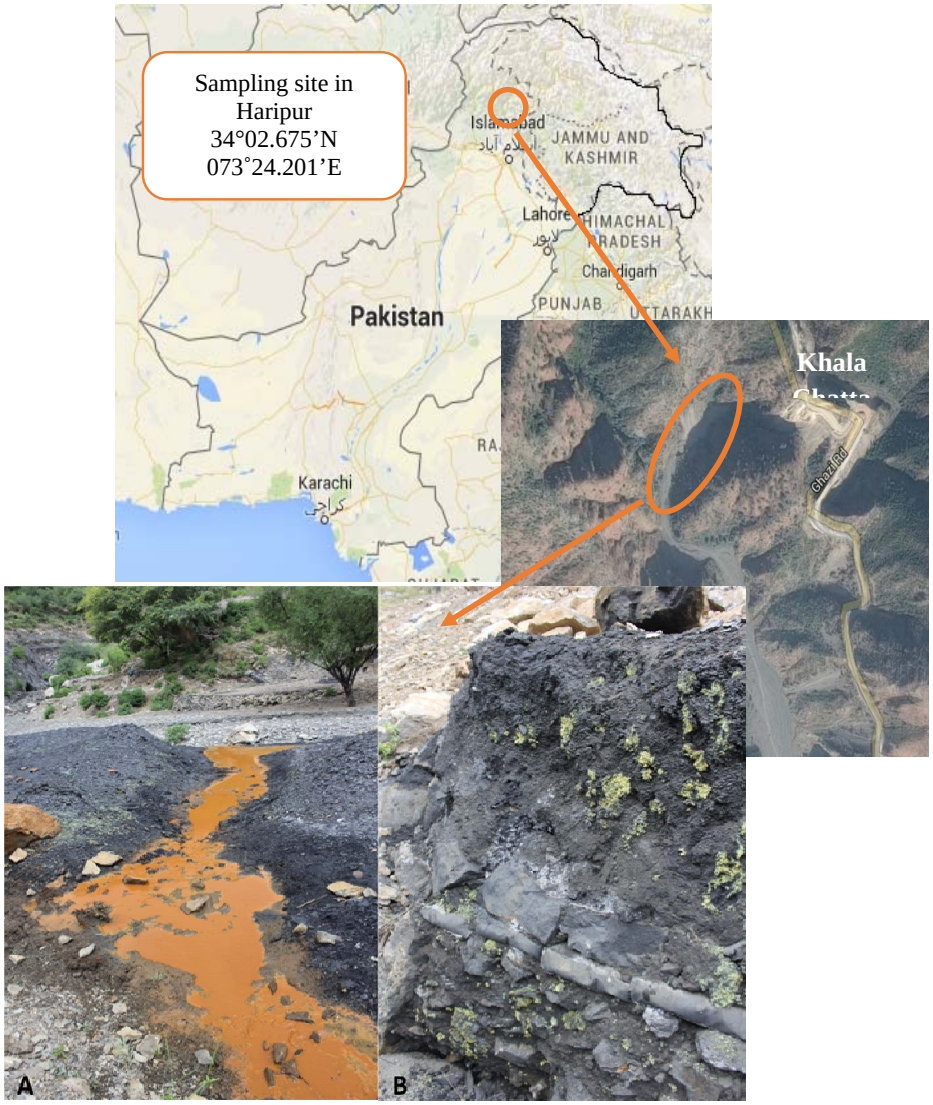

Fig. 1. Sites for collection of soil and water samples for isolation of sulfur oxidizing bacteria (A) Acid mine drainage; (B) Black shale.

Table 1. Characteristics of sampling sites and pH reduction and cfu/mL after 12 days incubation of samples.

No.	Samples	Location	pH of site	Temperature (°C) of site	pH after incubation	Cfu/mL
1.	BS_1	Soil sample collected from surface (removing 1 cm top soil) of black shale having yellow precipitates of sulfur	2.7	28˚C	0.8	1.5×10^7
2.	BS_2	Soil sample collected from deep portion of black shale having brown residues, and burning effect due to exothermic reaction	5.4	28˚C	1.3	1.3×10^5
3.	AMD_1	Liquid and residues sample collected from outside of mine, present high amount of ferric iron and iron hydroxide	6.1	25˚C	1.7	7.4×10^6
4.	AMD_2	Liquid and residue sample collected from inside of mine, cloudy and rusty color due to the presence of ferric iron and sulfur	4.7	20˚C	1.4	1.3×10^7

Isolation of sulfur-oxidizing bacteria: Sulfur-oxidizing bacteria (SOB) were isolated by inoculating water and soil samples collected from AMD and BS in an extremely selective basal salt medium containing thiosulfate as energy source and CO_2 as a sole source of carbon (Silverman & Lundgren, 1959). The composition of basal salt medium (g/L): [$(NH_4)_2SO_4$, 3.00; $MgSO_4.7H_2O$, 0.50; K_2HPO_4, 0.50; KCl, 0.10 and $Ca(NO_3)_2$, 0.012] dissolved and autoclaved separately in 900 mL distilled water having pH 4.5. 10.0 g of $Na_2S_2O_3.5H_2O$ was dissolved in 100 mL distilled water, sterilized separately by filtration (0.22 µm Millipore GVWP filters), and then added into basal salts solution. The suspension of AMD and BS samples was prepared in sterilized distilled water in Erlenmeyer flask on shaker incubator at 30°C and 150 rpm overnight. After incubation, 5% of suspension was inoculated in 95 mL of thiosulfate medium in Erlenmeyer flasks in separate and placed in shaker incubator at 150 rpm and 30°C for 12 days. The experiment was run in triplicate and control was run in parallel under similar conditions. The pH of culture was checked and colony forming unit (CFU) was calculated in samples collected after 12 days incubation by using solid thiosulfate media having agarose (0.5% w/v) as solidifying agent. SOB causes oxidation of sodium thiosulfate that results into decrease in pH of the medium due to acid production and the medium become turbid that confirms the growth of SOB. 1 mL of culture broth is transferred to fresh thiosulfate medium and re-incubated at 30°C and 150rpm for 12 days. This procedure is repeated 3 times and cells were harvested at 12000 rpm for 15 min at 4°C. The cells were re-suspended in sterilized distilled water and spread on solid thiosulfate medium plates having agarose as solidifying agent (0.5% w/v agarose sterilized separately in 100 mL distilled water and then added to basal salts and thiosulfate mixture as discussed above) and incubated at 30°C for 14 days. The plates were checked for bacterial growth after incubation, then sub-cultured and preserved in 20% glycerol at -80°C for long duration.

Quantification of sulfate ion: The concentration of sulfate ion (SO_4^{2-}) produced was determined by the method of Cha *et al*. (1999). Barium chloride solution (10% w/v) was added to culture supernatant in 1:1 and mixed up vigorously. A white turbid solution of barium sulfate was produced that was measured at 450 nm by spectrophotometer. Each sample was analyzed in triplicate and mean value of absorbance was used to calculate the (SO_4^{2-}) concentration. The values obtained were then compared with the sulfate standard curve (Kolmert *et al.*, 2000). For sulfate calibration curve potassium sulfate (K_2SO_4) was used by dissolving K_2SO_4 in deionized water to known concentration from 0 to 5 mM. The sulfate concentration is directly proportional to the turbidity of solution.

Optimization of culture conditions

Effect of temperature and pH on growth of SOB: The effect of culture conditions such as temperature ranges (15-40°C) and pH (1-9) on growth of SOB was determined in order to optimize these conditions. SOB were cultured at the above mentioned temperature and pH ranges in thiosulfate broth medium on shaker incubator (150rpm). The optical density (OD) was measured at 600 nm by spectrophotometer at 24 hours intervals.

Effect of substrate on growth of isolates: The growth characteristics of SOB were checked in the presence of different substrates as energy source such as anhydrous glucose (5%), $Na_2S_2O_3.5H_2O$ (1%) and Ferrous sulfate (5%) in $9KFe^{+2}$ medium with pH 2.0 (Silverman & Lundgren, 1959) and elemental sulfur (2%) in basal salt medium in separate flasks and incubated at 30°C and 150rpm in shaker incubator. The experiment was run in triplicate with a control in parallel under similar conditions. The results were interpreted by drop in pH in case of thiosulfate and elemental sulfur media, and turning media color to brick red in case of $9KFe^{+2}$ and turbidity in glucose medium. Cells were harvested after centrifugation at 12000 rpm for 10 minutes at 4°C, and inoculated on basal salts agar plates supplemented with glucose and thiosulfate on $9KFe^{+2}$ medium.

Identification of sulfur-oxidizing bacteria: All the isolates were presumptively characterized by morphological and microscopic examination. These were also subjected to various biochemical tests e.g., oxidase and catalase production, including other enzymatic activities and acid production from carbohydrates were determined by conventional methods (Holt, 1994). 6 isolates were selected on the basis of maximum sulfate ions production and phylogenetic analysis was done through 16S rRNA gene sequencing.

DNA extraction and phylogenetic analysis: Bacterial cells were harvested through centrifugation in the late exponential phase, and resuspended in 567 µL TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0). 3 µL proteinase k (2% w/v) and 30 µL of sodium dodecyl sulfate (10% wt/vol) was added to cell suspension, then mixed up and incubated at 37°C for 60 min. 100 µL sodium chloride (5 M) and 80 µL CTAB/ NaCl (10% w/v CTAB, 0.7 M NaCl) was added to cell suspension, and incubated at 65°C in a water bath for 10 min. Same volume of chloroform/isoamyl alcohol (24:1) was added to the mixture and centrifuged at 12,000xg for 5 min afterwards to precipitate out polysaccharides. The supernatant was collected and equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to it. The solution was thoroughly mixed and centrifuged at 12,000xg, the proteins were precipitated out and supernatant was collected. Isopropanol was added to the supernatant that precipitate the DNA. The mixture was centrifuged at 12,000xg for 5 min and removed the supernatant. The DNA was resuspended in 100 µL TE buffer having RNase and stored at 20°C for further analysis (Ausubel *et al.*, 1995).

16S rRNA gene sequencing was also performed for the identification of the bacterial isolate. The full-length gene was amplified from DNA using 27F' (5'-AGAGTTTGATCCTGGCTCAG-3') and 1494R' (5'-CTACGGCTACCTTGTTACGA-3') bacterial primers (Zeng *et al*., 2009). The 20 mL reaction mixture for PCR consisted of sample DNA 1 mL, PCR buffer 2 mL, deoxynucleotide triphosphate (dNTP) mix 2 mL, forward and reverse primer 2 mL each, ex taq DNA polymerase (Takara Shuzo, Otsu) 0.5 mL and distilled water 10.5 mL. At first, the reaction mixture was incubated at 96 C for 4 min. Then performed 35 amplification cycles at 94 C for 45s, 55 C for 60s, and 72 C for 60s. Reaction was further incubated for 7 min at 72 C. A positive control (*Escherichia coli* genomic DNA) and a negative control were included in the PCR. The PCR product was purified by using Montage PCR Clean up kit (Millipore) in order to remove unincorporated PCR primers and dNTPs from PCR products. The purified PCR products were sequenced by using 2 primers, 518F' (5'-CCAGCAGCCGCGGTAATACG-3') and 800R' (5'-TACCAGGGTATCTAATCC-3'). Sequencing was performed by using Big Dye terminator cycle sequencing kit v.3.1 (Applied Bio-Systems, USA). Sequencing products were resolved on an Applied Bio-Systems model 3100 automated DNA sequencing system (Applied Bio-Systems, USA) at the Macrogen, Inc., Seoul, Korea.

The sequences obtained in this study were examined for chimeras by the check-chimera program of the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) and these sequences were also compared with the 16S rRNA gene sequences deposited in public database GenBank (NCBI) using the BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/) based on single sequence for each isolates. The sequences of 16S rRNA gene of various bacteria (including those closely related to the unknown sequences, as indicated by the BLAST search) obtained from the GenBank database were aligned with the new sequences using BioEdit 6.0. The phylogenetic tree was constructed by the Maximum Likelihood method with robustness of 1000 bootstrapping value in MEGA 6.0 (Tamura & Nei, 1993). All the sequences obtained were submitted to NCBI GenBank and the accession numbers have been assigned.

Statistical analysis: The data was analyzed by using Excel data sheets and GraphPad, version 5.00 for windows (GraphPad Software, San Diego California USA, www.graphpad.com). Sulfate quantification and samples analysis for various metals was determined in triplicate and reported as a mean value ± SD (Steel *et al*., 1997). Two way analysis of mean (ANOVA) was applied to find out significant difference between concentration of metals and sulfate. Statistical significance was defined as $p<0.05$.

Results

Sample analysis for metals: The mean concentrations (mg/Kg and mg/L) of 13 metals such as calcium, magnesium, chromium, cobalt, nickel, zinc, copper, lead, iron, cadmium, potassium, manganese and sodium are presented in Table 2, showing mean values ± SD. Mean concentration of all the studied metals in samples were higher than the control as a reference and permissible range determined by Environmental Protection Agency (EPA) and the World Health Organization (WHO) of international standard for soil and water. The mean value difference of all the metals in the samples and control were observed (ANOVA/LSD) for P-value ($p<0.05$).

Isolation of sulfur-oxidising bacteria: In this study, a total of 24 bacteria were isolated from BS and AMD that could oxidize sulfur in thiosulfate broth indicated by decrease in pH, termed as sulfur oxidizing bacteria (SOB). Among these 24, SOB, 10 isolates from BS (BS1-BS10) and the remaining 14 isolates were isolated from acid mine drainage (AM1-AM14). All these SOB could reduce the medium pH even below 1 within 12 days of incubation. The pH and temperature of sampling sites and reduction in pH and cfu/mL after 12 days of incubation was mentioned in Table 1.

Quantification of sulfate ions: The sulfate ions assay was based upon the sulfate ion precipitation with barium chloride. Among all the isolates from BS and AMD, strain BS3 and AM2 produced the maximum concentration of sulfate ions of 272 ± 8.67 and 268 ± 10.47 mg/L respectively and reduced the medium pH to 0.9 ± 0.03 and 1.0 ± 0.9 respectively from initial pH 4.5, however strain AM4 produced 179 ± 5.7 mg/L of sulfate and pH reduced to 1.9 ± 1.0, that are least amount reported in present study. The sulfate ion production ability and pH reduction of the remaining strains were in the following order mg/L, (pH reduction), AM11; 258 ± 18.30 (1.1 ± 0.6), AM14; 258 ± 10.9 (1.1 ± 0.4), AM1; 253 ± 6.26 (1.1 ± 0.9), AM3; 250 ± 11.6 (1.1 ± 0.8), BS1; 248 ± 8.06 (1.5 ± 0.4), BS5; 246 ± 16.8 (1.3 ± 0.7), BS10; 243 ± 14.4 (1.2 ± 0.8), BS7; 241 ± 6.30 (1.2 ± 1.0), AM10; 239 ± 8.9 (1.4 ± 0.7), BS2; 235 ± 8.18 (1.6 ± 0.2), BS6; 235 ± 5.08 (1.4 ± 0.1), AM12; 233 ± 11.2 (1.2 ± 0.5), BS8; 227 ± 8.27 (1.1 ± 0.8), AM7; 227 ± 11.39 (1.7 ± 0.6), BS9; 223 ± 8.36 (1.4 ± 0.8), AM5; 223 ± 21.06 (1.4 ± 0.6), AM6; 220 ± 5.3 (1.6 ± 0.2), AM13; 218 ± 10.7 (1.4 ± 0.4), AM8; 206 ± 9.5 (1.8 ± 1.0), BS4; 191 ± 8.3 (1.8 ± 1.1), AM9; 191 ± 4.4 (1.9 ± 1.0). The sulfate production efficiency of all 24 SOB isolates showed that they produce significantly high amount of sulfate ion from sodium thiosulfate supplemented in the medium.

Table 2. Concentration of metals in samples collected from black shale (BS) and Acid Mine Drainage (AMD).

	Metals concentration in Soil (mg/Kg) and Water (mg/L)												
Sample	**Nickel**	**Manganese**	**Sodium**	**Potassium**	**Cadmium**	**Cobalt**	**Zinc**	**Copper**	**Iron**	**Magnesium**	**Chromium**	**Lead**	**Calcium**
BS_1	26.5±1.36*	4.5±0.01*	3000±317.5*	297±5.3	43.5±3.0*	93.0±3.0	86±1.8*	27.6±2.4	54.4±2.8*	128.2±11.5	25.5±2.5	36±3.7	510±18.0
BS_2	24.5±1.54*	4.7±1.17*	3261.5±135.8*	389±44.8	45.5±4.2*	64.4±2.9	103±3.3*	45.9±2.2	56.5±8.5*	169.4±5.8	40.9±4.6	57.5±1.5	362.8±12.1
Soil control	10±1.1	0.032±0.01	112.8±34.3	25±3.9	1.03±0.78	0.81±0.02	39±5.44	4.7±1.41	9.3±1.22	1.02±0.66	8.7±1.65	8.14±1.9	121.6±4.8
Soil tolerable range	10	3.3	70	70	0.02	4.4	18	10	32.7	22.9	1.3	2	141
AMD_1	14.5±2.3*	4±0.06*	2972.8±197	79.41±15.3	47.15±4.33*	51.4±2.44	141±4.59*	44±2.3*	73±4.1*	127±4.3*	56±2.7	65.5±1.3	360±4.9
AMD_2	16±2.02*	5±1.11*	2808.5±96.9	124.6±14.5	49.25±4.27*	63.3±6.00	191±8.08*	60±4.2*	87±7.1*	111±5.6*	70.5±4.6	57±1.1	233.5±2.4
Water control	9±1.08	0.12±0.02	2711.1±21.8	2.62±1.54	18.6±2.7	16.85±2.2	5±1.1	1.2±0.02	35±4.4	68±5.3	4.09±1.9	26.5±1.5	14.6±1.2
Water tolerable range	0.2	0.4	50	20	0.01	0.05	3	0.2	0.3	50	0.1	5	75

Values are mean values ± SD of readings investigated individually in triplicate

Permissible Range = WHO, Guidelines for drinking water quality, 2008, Permissible limits of heavy metals in soil and plants, 1996 and International year of fresh water, 2003. EPA, Risk Assessment Guidance for Superfund, Human Health Manual, 1985

*Significant at $p<0.05$

Table 3. Morphological, Nutritional and Biochemical Characterization of six best sulfur-oxidizing bacteria from BS and AMD.

S. No.	**Characteristics**	**AM1**	**AM2**	**AM3**	**AM11**	**AM14**	**BS3**
1.	Morphology	Rods	Short rod	Short rod	Coccobacilli	Short rod	Short rod
2.	Gram staining	-	-	-	-	-	-
3.	Medium supplements						
	Glucose	+	+	-	+	+	+
	Elemental sulfur	+	+	+	+	+	+
	Sodium thiosulfate	+	+	+	+	+	+
	Ferrous sulfate	+	+	+	+	+	+
4.	Nutritional type	Mixotrophs	Mixotrophs	Autotrophs	Mixotrophs	Mixotrophs	Mixotrophs
5.	Biochemical tests						
	Starch	-	+	-	-	+	-
	Gelatin	+	+	+	+	+	+
	Casein	+	+	+	+	+	-
	Citrate	+	+	+	+	+	+
	Urease	+	+	-	+	-	+
	Indole	-	-	-	-	-	-
	Methyl red	-	-	-	-	-	-
	VP	-	-	-	-	-	-
	Catalase	+	+	+	+	+	+
	Oxidase	-	-	-	-	-	-
	Motility	+	+	+	+	+	+
	Triple sugar iron						
	Slant	A	A	K	A	A	A
	Butt	A	A	K	A	A	A
	H_2S	-	-	-	-	-	-
	Gas	-	-	-	-	-	-
6.	Identified organisms	*Alcaligenes aquatilis*	*Alcaligenes aquatilis*	*Pseudomonas putida*	*Bordetella petrii*	*Bordetella petrii*	*Stenotrophomonas maltophilia*
7.	16S rRNA similarity index	91%	95%	98%	99%	99%	99%

Legend - = Negative results; +, Positive results; A, Acidic (yellow); K, Alkaline (red)

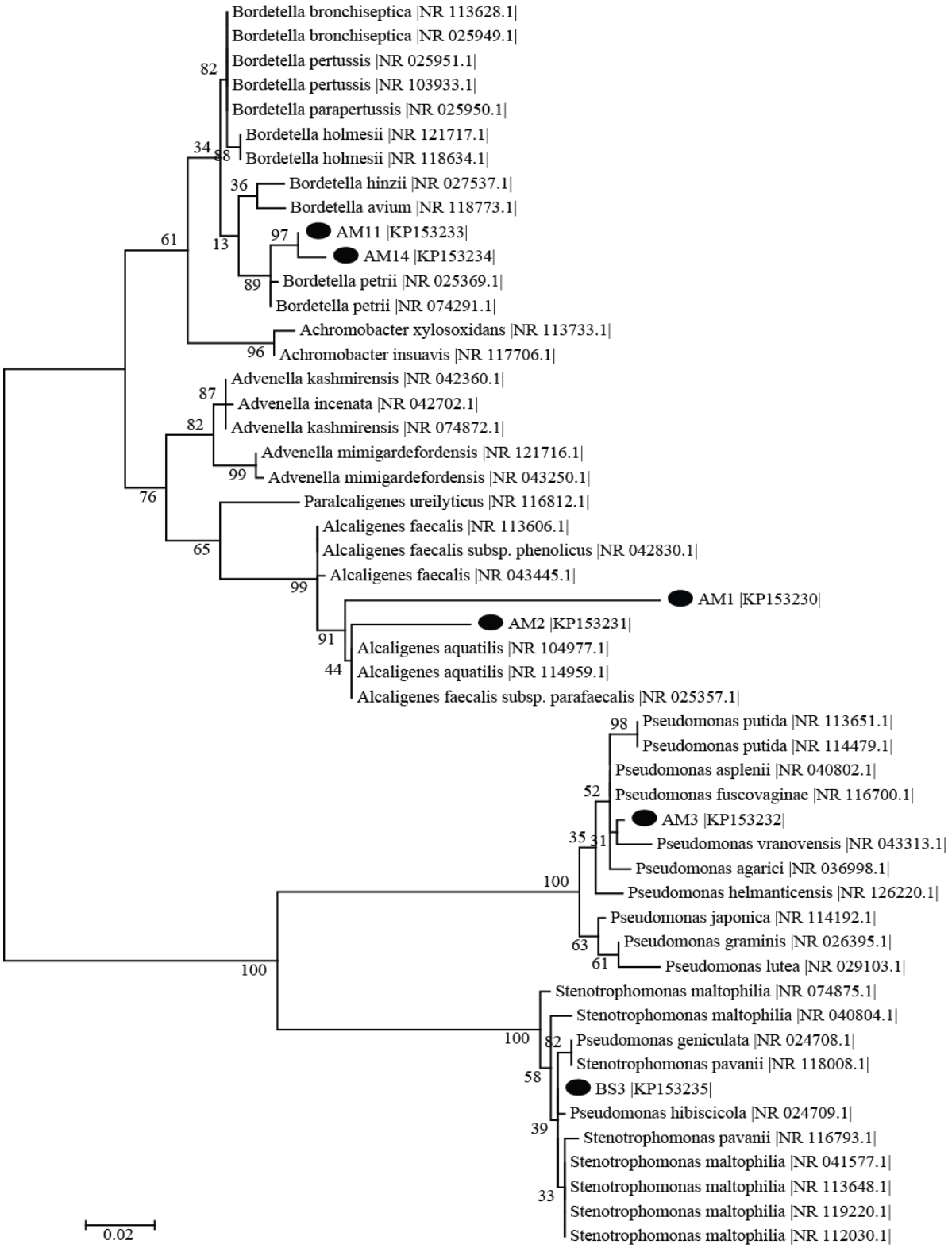

Fig. 2. Molecular Phylogenetic analysis of sulfur oxidizing strains by Maximum Likelihood method based on analysis of 16S rRNA gene sequences in relation to reference sequences from the GenBank database. The tree with the highest log likelihood (-2616.7217) is shown. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The analysis involved 51 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 505 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. The scale bar represents 1 nucleotide substitution per 100 nucleotides of 16S rRNA sequence. Bootstrap values of 1000 are shown.

Characterization of sulfur-oxidizing bacteria: The entire SOB were characterized on the basis of their cultural and nutritional characteristics. All the SOB were gram negative, and produced tiny smooth white, off white to yellowish colonies on thiosulfate agar with a peculiar sulfur odor and clear zone. The bacteria could optimally grow at temperature fall in mesophilic range of 25°C in case of AM11, 30°C in case of AM1, AM2, AM3 and AM14 and 35°C in case of BS3 and pH range fall in extreme acidophiles in case of AM2, AM3 and AM14, moderate acidophiles in case of AM11 and BS3 and acid-tolerant in case of AM1. All isolates were either autotrophic or mixotrophic. All the SOB were identified through conventional biochemical tests. Characterization of six best sulfate producing isolates are shown in Table 3.

Six isolates named AM1, AM2, AM3, BS3, AM11, and AM14, that produced highest amount of sulfate ion were selected for phylogenetic analysis through 16S rRNA gene sequencing. Sequences from these 6 strains showed variable similarities with the reference sequences from NCBI GenBank. Strain AM1 and AM2 have 91% and 95% sequence identity with two strains of *Alcaligenes aquatilis* [NR114959] and [NR104977], respectively. Strain AM3 was found with 98% sequence similarity with *Pseudomonas putida* [NR113651], BS3 with 99% with *Stenotrophomonas maltophilia* [NR041577] and isolates AM11 and AM14 were identified *Bordetella petrii* [NR074291] [NR025369] with both 99% sequence similarity (Fig. 2).

Nucleotide sequence accession numbers: The nucleotide sequence reported here can be obtained from NCBI nucleotide sequence database under accession numbers KP153230 (AM1), KP153231 (AM2), KP153232 (AM3), KP153233 (AM11), KP153234 (AM14) and KP153235 (BS3).

Discussion

Although much of the research work has been done on microbial diversity of both black shale (BS) and acid mine drainage (AMD). However, to the best of our knowledge, no significant study has been reported in Pakistan. In the current study, a total of 24 isolates were obtained in pure culture from samples of BS and AMD. The method of isolation in both liquid and solid thiosulfate medium was extremely selective and would not typically captured the majority of iron oxidizing bacteria like *Acidithiobacillus ferrooxidans* and the sulfur oxidizing bacteria such as *Acidithiobacillus thiooxidans* that would be missed with this technique. All these isolates efficiently oxidized sulfur and sulfide into sulfate ion and resulted into reduction in pH of thiosulfate broth medium up to 1.0 from the initial pH 4.5 after 12 days of incubation. Various researchers have already discussed in their reports that decrease in pH is due to the oxidation of sulfur to sulfuric acid in the medium by sulfur-oxidizing bacteria (Khalid *et al*., 1993; Donati *et al*., 1996; Vidyalakshmi & Sridar, 2007). The environmental pyrite (FeS_2) and other sulfides are oxidized quickly by the presence of oxygen and/or ferric iron and the activity of these acidophilic microbes. Williamson & Rimstidt (1994) reported that the abiotic rate of pyrite oxidation rose with increase of oxygen concentration with decrease of pH. Sulfide minerals oxidation in natural environment increases with decrease of pH into a range that encouraging bacterial oxidation of ferrous iron (Singer & Stumm, 1970; Kleinmann *et al*., 1980; Nordstrom, 1982). The bacteria facilitated rate of pyrite oxidation by ferric iron is 2-3 fold faster than abiotic oxidation by oxygen at low pH 2.0 (Nordstrom & lpers, 1999).

Among SOB, strain BS3 and AM2 produced maximum amount of sulfate ions, i.e., 272 and 268 mg/L, respectively. The production of sulfate ions by *Thiobacillus* spp has already been reported in the range of 40 mg/L at different sulfide minerals concentration (Ravichandra *et al*., 2007). Highest concentration of sulfuric acid production was reported for strain ATTC55128 (243 mg/L) and AHB436 (230 mg/L) (Babana *et al*., 2011). The sulfate ion concentration produced by strain ASWW-2 was approximately 30 g/L (Lee *et al*., 2000). It is possible to achieve maximum sulfate yield from *T. thiooxidans* under optimal growth conditions by using factorial design and response surface methodology (Liu *et al*., 2004). Several researchers have reported sulfur-oxidizing *Thiobacillus* spp. that could utilize both thiosulfate and elemental sulfur and reduce the pH from 5.6-6.2 to 2.6-2.8 (Waksman & Joffe, 1922; Vidyalakshmi & Sridar, 2007). Our strains not only oxidized sulfur but also iron and glucose as energy source and considered as mixotrophs. This sulfur and iron oxidation ability enables them to use for extraction of metals from low-grade ores where the ferric iron act as an oxidizing agent. AMD generation occurred due to dissolution of sulfide ores, and the ores oxidation rate enhanced several folds by sulfur and iron oxidizing bacteria and archaea (Baker & Banfield, 2003). Most notably organisms in AMD generation are chemolithotrophic sulfur and iron oxidizing acidophiles (Bond *et al*., 2000; Druschel *et al*., 2004; Kock & Schippers, 2008). All man-made and natural acidic environments are commonly dominated by these chemolithotrophic bacteria. Unexpectedly in the black shale and acid mine drainage of Khala Chatta, Haripur, mixotrophic acidophiles were more abundant in numbers than autotrophic acidophiles. Enumeration of bacterial isolates from enrichment culture of BS and AMD were accomplished effectively through conventional plating methods with agarose as hardening agent. Samples from AM_2 had high CFU/mL of 1.3×10^7 as compared to other samples. Subsequently, it was discovered from the work that the bacterial strains reported from AMD are mixotrophs and consumed both organic and inorganic substrates as a source of energy. Majority of these mixotrophs were isolated from the resource restrictive environment of acid mine drainage where there is no sufficient amount of organic matter and light for ideal growth of chemoheterotrophs and phototrophs respectively, however mixotrophs can use both the organic and inorganic resources synchronously for ideal growth and viable in both conditions. The same BS and AMD environments were comparatively study for heterotrophic and mixotrophic acidophiles and found rich of mixotrophic bacteria by Sajjad *et al*., 2015. Extreme acidic environments hold low concentration of dissolved

organic compounds (DOC) for the optimal growth of heterotrophs and only the autochthonous organic matter originates from cell lysates of chemoautotrophic acidophiles acts as a source of organic matter (Ñancucheo & Johnson, 2010). The study carried out by Nixdorf *et al.* (1998) also confirmed the shortage of organic material in extreme acidic mining lakes. It is experimentally proved that mixotrophic flagellates compete with obligately heterotrophic flagellates for food uptake and only the mixotrophic strategy become effective especially in resources limiting area (Rothhaupt, 1996).

Sulfur-oxidizing bacteria were identified as *Pseudomonas*, *Stenotrophomonas*, *Alcaligenes*, *Bordetella* spp and *Thiobacillus* spp. Sulfur oxidizing *Pseudomonas* spp. have also been isolated from soil of Bhitarakanika, Odisha, India (Thatoi *et al*., 2012) and mangrove soil of Mahanadi River Delta (Behera *et al*., 2014) that produce sulfate ion. National center of biotechnology information (NCBI) also contain sequences with similar index having Accession No [KF896128], isolated from metal contaminated sediments and Accession No [KC749159] isolated from acid mine drainage of Tong Ling pyrite mine, Anhui Province, China. Preston *et al*. (2014) reported *Stenotrophomonas* spp from acidic environment of marine archeological timbers and reported 85% and 87% of clone library similar with *Stenotrophomonas* spp at pH 3.0 and pH 1.7, respectively. *Alcaligenes* spp. have been reported during the characterization of microbial population of wetland receiving acid coal mine drainage (AMD) (Nicomrat *et al*., 2006) and acidic, geothermal springs of Montserrat as fourth most common clone type (Burton & Norris, 2000).

The concentration of various metals in all the samples were found higher than reference sample and the permissible limit set by WHO and EPA. Normally black shale are deposits of huge amount of metals that are extracted with different methods depending on the amount of metals present. Tariq *et al*. (2013) reported a significant amount of various metals in black shale of Chimiari Khyber Pakhtunkhwa region that fall in the same range with Khala Chatta. Anjum *et al.* (2010) also reported high amount of the heavy metals from black shale in Pakistan. Various geological surveys in Pakistan confirmed the presence of aluminum, arsenic, gold, copper, silver, iron, uranium and zinc etc., which mostly occur as low grade ores (Ilyas *et al*., 2012). Metals spread in water reservoirs depending upon certain factors like mobility and solubility of each metal, ground water, surface runoff, dissolution of metals from sediments and deposition from the atmosphere affect metallic levels (Jung, 2001, Jian-Min *et al*., 2007; Jung, 2008). In present study the level of metals was very high in water effluents from mines due to the action of microbes and water in the presence of oxygen. The rain or ground water comes in contact with mines tailing decrease in pH and cause acidity of mines soil making more metals available and soluble in water.

Conclusions

The present study emphasizes on isolation and characterization of sulfur-oxidizing bacteria from black shale and acid mine drainage from Khala Chatta, Haripur, KPK, Pakistan. Characterization of isolates from specific environment is not only important to understand the role of isolates in the niche but also to understand the condition and requirements for their survival in these niches. The present finding showed the presence of obligate autotrophs and mixotrophs in low pH habitats that have high potential to reduce pH of the culture medium and efficiently generate sulfate ion. This characteristic of sulfur-oxidizing bacteria can be utilized for the purpose of bioleaching of metals from low-grade ores. Several geological surveys in Pakistan confirmed the presence of aluminum, arsenic, gold, copper, silver, iron, uranium and zinc etc., which mostly occur as low grade ores, so conventional metallurgical techniques are not fit for their extraction for developing country like Pakistan. This pH reducing property of sulfur-oxidizing bacteria enable them to use for reclamation of alkali soils. They can also be applied to decrease environmental pollution and promotes sustainable agriculture. Further investigation on microbial diversity of these ecosystems will be worthy to understand the mechanisms due to which these acidophiles survive in such an extreme environment, antibiotic production, potential biotechnological compounds like acid tolerant proteins and enzymes and biogeochemical process.

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