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Isolation and Molecular Characterization of Two Bacterial Strains with Higher Bioleaching Potentials, from Agbaja Mines (Kogi State, Nigeria)

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Bacterial strains with higher bioleaching capacity (BC) are relevant for the mining industry, being more eco-friendly methods, for instance more than cyanide based extraction. Microbiological samples were collected from selected sites in Agbaja Mines, which after a growth enrichment step, cultures were screened for iron BC, including chemical quantification of iron species. Strains with the highest BC were morphologically, biochemically and molecularly characterized. Two bacterial strains with iron BC *Leptospirillum* sp. *And Acidithiobacillus* sp. (AGBAJA-1 and AGBAJA-2, respectively) were isolated from Agbaja Mines of Kogi State Nigeria. Bioleaching studies carried out shows that prior to inoculation of the iron ore solution with the organisms, raw Agbaja iron ore had a concentration of 25.10 mg/g iron while Zinc, Copper and Lead were 1.3903 mg/g, 0.094 mg/g, and 0.2813 mg/g respectively from X-ray diffraction analysis. The degree of bioleaching as a function of the amount of iron leached into solution was higher (in four weeks bioleaching test) than the other metals. Molecular characterization by sequencing the 16S ribosomal unit, amplification with universal primers, yielded high quality amplicons that were sequenced. Their phylogenetic/Blast comparison showed more than 97% relationships with *Acidithiobacillus*

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ferroxidans ATCC 23270 and *Leptospirillum ferrovirans DSM 15468,* families of bacteria in the National Centre for Biotechnology Information (NCBI) data bank. These are moderately thermophilic acidophilic bacteria that may represent an opportunity for developing a greener iron extraction process.

Keywords: Bioleaching; molecular characterization; iron ore; mining; Kogi-Nigeria.

1. INTRODUCTION

The role bacteria and their metabolites play in the mineral processing industry is becoming more popular. In recent times microorganisms are widely used in the leaching of mineral oxides and sulphides and the remediation of wastes from industrial contaminated environments [1]. Due to increasing demand for minerals and the depletion of high grade ones, mineral research is increasingly focusing on the beneficiation of low grade ores to produce materials suitable for a global market. The industry's focus on reducing environmental impact of mining and mineral processing has led to significant advances in the application of biotechnology in the sector [2]. This may be of particular importance to the iron ore mining and steel making industries where an increasing worldwide demand for steel and depleting resources of high grade iron ores mean that lower grade deposits will soon need to be mined to meet up with market demands. Several conventional beneficiation processes, including flotation and flocculation, have been successfully integrated into a number of iron ore processing operations in the USA and Brazil [3]. Often, the efficiency of these conventional treatment processes is highly dependent on the ore composition and nature, meaning that one particular process may not necessarily be universally applied to all mineral deposits [4]. In the future, as high grade reserves are depleted, the iron ore industry will need to embrace cost effective, environmentally benign treatment processes for the beneficiation of these lower grade ores.

A biological approach may be one such option. Activities of microbes are very important in the turnover of organic and inorganic matter on earth. Neutrophilic bacteria associated with ferric (Fe^{3+}) oxide precipitation have been known for a long time. Some species were shown to precipitate ferric (Fe^{3+}) oxides during heterotrophic metabolism. Straub et al. [5] suggested that bacterial Fe^{3+} iron oxidation promotes coupling between iron oxidation and reduction by producing amorphous or poorly crystalline ferric (Fe^{3+}) oxides, which are readily available for ferric (Fe^{3+}) reducing bacteria such as *Thiobacillus ferrooxidans, Sulfolobus* sp. and *Bacillus megaterium*. It has now been realized that organisms which are capable of transforming metals and inorganic compounds such as iron, copper, uranium, sulfur, and manganese can be utilized not only for extracting minerals from poor ores but at the same time with careful planning they can also be utilized for environmental management, such as by removing excess iron from water bodies and similar environments [5]. Bioleaching is gradually becoming an important tool among the available mining technologies where it is no longer a promising technology but an actual economic alternative for treating specific mineral ores [6]. Examination of current large-scale bioleaching operations reveals that a number of these activities are undertaken in developing countries [7]. This is not purely accidental but necessarily as a result of two important factors. first, Many developing countries have significant mineral reserves and mining constitutes one of their main sources of revenue. Secondly, bioleaching is a technique especially suitable for developing countries like those in sub-Saharan Africa because of its simplicity and low capital cost requirement [8]. For example by the year 2000 more than 50% of the world copper production was recovered by this process. This explains the importance of bioleaching for extracting low grade mines in developing countries.

New insights into the physiologies of iron-oxidizing proteobacteria are emerging as increasing numbers of their genomes are sequenced and annotated. As with other bacteria, advances in genomics, transcriptomics and proteomics technologies have helped to understand the mechanisms involved in the metabolism of iron-oxidizing proteobacteria. Today, it is a completely different scenario compared with the year 2000, when the first gapped genome sequence of *Acidithiobacillus ferrooxidans* (formerly *Thiobacillus ferrooxidans* and the archetype of a bioleaching microorganism) was published. Currently, 55 bacterial and 36 archaeal complete genomes from microorganisms that are present in

bioleaching processes are publicly available according to the NCBI database, and the tendency to an exponential increase is evident. One main question is how the knowledge generated in these 15 years of genomics of leaching microorganisms has contributed to improvements in this field and particularly, which are and would be the major future impacts at industrial scale. Genomics has certainly made a major impact on our knowledge of bioleaching. First of all, partial and full genome sequencing has allowed the determination of biodiversity within leaching environments and the development of molecular-based methods to scrutinize the temporal population dynamics of different bioleaching processes. For a long time, *Acidithiobacillus ferrooxidans* was thought to be the most significant microorganism for metal sulfide bioleaching, however, advances in genomics knowledge together with the development of bio-identification molecular techniques such as gel electrophoresis and quantitative PCR (qPCR) has driven the exploration of extreme mineral leaching environments for new microorganisms with potential commercial applications. This gives a more comprehensive understanding of the biodiversity of acidophilic environments. Studies on the molecular genetics of most biomining microorganisms are still not enough even with one or two genes from several bacteria cloned [9]. The area of biomining still has a number of questions that are begging for answers. Despite the encouraging incentives from bioleaching as an alternative to chemical leaching for metal recovery, there are still great challenges with these processes at molecular level. The aim of this current work therefore was to perform some molecular characterization of isolated bacteria with potentials to leach iron ore from Agbaja mining site of Kogi State, Nigeria. This is with view to generating information for future molecular manipulations and possible applications in Nigeria.

2. MATERIALS AND METHODS

2.1 Equipment

Some of the equipment used in this work include Intelligent Thermostatic Shake Cultivation Cabinet (incubator shaker, thermo scientific) made in USA, Vertical Heating Pressure Steam Sterilizer (autoclave-Forge sterilimatic ,USA), Colony Counter from Biorad, Gallenkamp Laboratory Oven, Zymo Research Fungal/Bacterial DNA Extraction Kits™ (Cat. # 6001), Alpha Innotech Gel Documentation Machine, (Kodak 4000R), Sigma_Aldrich SHU6 Electrophoretic tank, Haraeus multifuge XIR entrifuge, Labconco purifier class II Biosafety cabinet, Vortex machine, Peltier-based thermal cycler (Biorad), and ABI prism Sequencer 310.

2.2 Collection of Samples from Agbaja Mining Area

In this work, Agbaja iron ore mining site of Kogi State was selected for ore collection. The samples were collected from different parts of the mine, including the different parts of surface of the mine and depth of 3-5 m deep. Samples included the rocks and concentrates. The samples were collected under aseptic condition with the use of sterilized plastic bags and immediately transported to the Laboratory. The iron ore materials were milled and separated into different particle size ranges, namely 0.22 mm - 0.84 mm (particle size), by sieving. Separation according to size was followed by treatment of the iron ore materials with 0.1 M hydrochloric acid (HCl) for 24 hours to remove exchangeable bases. The samples were then washed repeatedly with distilled water and adjusted to a pH of 2.0.

2.3 Geological Analysis of the Ore Sample (AOAC, 2000)

Analysis of the ore was carried out using an X-ray diffraction (XRD) and X-Ray fluorescence (XRF) machines to find out the contents of different elements within the ore.

2.4 Isolation of Microorganisms [10]

For isolating acidophilic microorganisms from the mine, a modified 9-K media was used. The media was prepared in four parts in the following way: $Na_2S_2O_3.5H_2O$ (2g) was added to 10ml of H_2O (Solution A), $FeSO_4.7H_2O$ (2g) was added to 10 ml of distilled water adjusted to pH 1.8 with 1 N H_2SO_4 (solution B), $(NH_4)_2SO_4$ (3g), KCl (0.1 g), $MgSO_4.7H_2O$ (0.5g), and K_2HPO_4 (0.5g) were dissolved in 500 ml of distilled water and adjusted to pH 3.4 with 1 N H_2SO_4 (solution C) and agarose (agarose for routine work from Sigma) (0.8 g) was added to 480 ml of distilled water (solution D). Solutions A and B were filtered and sterilized but solutions C and D were autoclaved. All solutions (A, B, C and D) were

mixed and the final pH was adjusted to 2.0. In this work, 100 ml of each medium was transferred into 250 ml conical flasks with the pH adjusted to 1.5, and autoclaved at 121 ^{o}C for 15 minute. One millitre of a filter sterilized $FeSO_4.7H_2O$ solution was added to each flask. Different concentration of mine samples including rocks and concentrates (0.5, 1, 1.5 and 2% w/v) were added to each flask. Incubation was carried out for three weeks on a shaker incubator at 30 ^{o}C and 180 rpm.

2.5 Screening the Isolates

Screening the isolates in order to check their ability for oxidizing ferrous iron to ferric iron, the 9K media was mixed with 6 g/l ferrous iron and about 100 µl of bacterial suspension added to the solution. The oxidation of ferrous iron to ferric iron was measured in form of absorbance values with a colorimeter. To plot the standard curve of ferric iron concentration, 0.1 ml of aqueous ferric sulfate solution was mixed with 3 ml of 10% 5-Sulfosalicylic acid (SSA) followed by addition of 97 ml of deionized water and absorbance measured at 500 nm. The amount of ferric iron in the test sample was calculated by extrapolating from the standard curve. Organisms with higher iron oxidation were then selected for further morphological and biochemical identification.

2.6 Identification of Bacteria

The preliminary identification of the isolated organism was carried out by performing Gram staining, motility test, oxidase and catalase tests. The pH and temperature range of bacterial growth and the ability for oxidizing different substrates (iron and sulphur) were also determined.

2.7 Growth of Iron Oxidizers (Edward et al., 2003)

The liquid media for the growth of iron oxidizers was made up of these combinations in g/L: $MgSO_4.7H_2O$, 0.50., $(NH_4)_2SO_4$ (0.15), KCl (0.05), KH_2PO_4 (0.05), and Ca $(NO_3)_2$ (0.01). The solution pH was adjusted to 2.0 using sulphuric acid and autoclaved at 121°C for 15 minutes. Filter sterilized ferrous sulphate solution of 0.5 micron (freshly prepared) was added to the solution to a final concentration of 50 mM, before inoculation and after inoculation flasks were adjusted in orbital shaker having temperature at 28°C. The isolate was maintained in nutrient agar slants and kept in the refrigerator for subsequent use.

2.8 Microbial Leaching (Bioleaching) Studies

Four grams of the crushed and sieved iron ore were transferred into six sterilized McCartney bottles containing 10ml of distilled water to five bottles and inoculated with a wireloopful of the bacteria isolates. The bottle without the innocular serves as control. All the bottles were incubated at 37^{o}C for different number of days per tube over a period of four weeks. At the end of a particular day(1-5 days) for a given bottle, the solution was filtered, washed with 2% Sodium dodecyl sulfate (SDS) and the residue dried. The samples were taken for elemental analysis using atomic absorption spectrophotometry (AAS) to determine the degree of bioleaching of the following metals (Fe, Zn, Cu, Pb and Ni) by determining the amount of iron leached out from the un-innoculated one using the formular below:

%Fe(II)= Mass of Fe before inoculation/Mass of Fe after inoculation x100

2.9 Elemental Analysis

Mineral content was determined by Association of Official Analytical Chemists methods (AOAC, 2000) using the atomic absorption spectrophotometer (AAS), *iCE* 3000 series. One gram of the sample was weighed and digested with nitric acid. After digestion, it was made up to 100ml mark in a volumetric flask with deionzed water. The absorbance of the sample was read directly on the AAS. Working standards of copper (Cu), nickel (Ni), lead (Pb), zinc (Zn) and iron (Fe) were prepared from stock standard solutions (1000 ppm) in 2M HNO_3. The absorbance was noted for each of the working standard solutions of the elements and samples using atomic absorption spectrophotometer (AAS). The calibration curves obtained for concentration versus absorbance was plotted. Data were statistically analyzed using fitting of straight line by least square method. A blank reading was also taken and necessary corrections were made during the calculation of the various elements.

2.10 Molecular Characterization of Cultured Organism

DNA of microbial cells was extracted using a ZR fungal/Bacterial DNA Miniprep ^{Tm} kit according to the manufacturer's instructions. Between 50-100 mg of bacterial cell isolate were scooped from the agar plate and added to 750 µl of lysis buffer, vortexed for 5 minutes, and centrifuged at 12,000 rpm for 2 minutes. The supernatant was transferred into a Zymo-spin^{TM} Spin filter then centrifuged at 10,000rpm for 2 minutes. Bacterial DNA binding buffer (200 µl)was added to the filtrate. This mixture was then transferred to a Zymo-Spin^{TM} IIC tube and centrifuged at 12,000rpm for 2 minutes. This step was repeated and the resulting filtrate was discarded. DNA pre-wash buffer was introduced to the tube followed by centrifugation at 12,000 rpm for 2 minutes. The fungal bacterial DNA wash buffer was added then the mixture was centrifuged at 12,000 rpm for 2mins. Using a clean 1.5 ml micro tube, 100 ul of the DNA elution buffer was added. This mixture was centrifuged at 12,000rpm for 1 min to precipitate the DNA.

2.11 PCR Amplification

The genotype identification of the microorganisms was performed using primers; 27F (5^1-GAGTTTGATCCTGGCTCAG-3^1) and 1492R (5^1-GGTTACCTTGTTACGACT-3^1), representing the forward and reverse primers. The PCR reaction was done in a 25µl reaction mixture containing 2.5 µl of 10X buffer supplemented with 15 mM $MgCl_2$, 100 mM Tris-HCl, 500 mM KCl, 0.5 µl of each oligonucleotide primer (F+R), 2µl of 0.04 mM each dNTPs, 2 µl of the extracted DNA, 0.25 µl of 0.25 units/25µlTaq DNA polymerase and 7.25µl of autoclaved distilled water. The reaction was run in a thermal cycler (GeneAmp®PCR System 2400, Perkin Elmer, U.S.A.) by heating at 95^{o}C for 5 min, and then amplification was performed with 35 cycles at 94°C for 2 min, an annealing step at 52^{o}C for 1 min as well as an elongation step at 72^{o}C for 2 min. Amplification ended with an elongation phase at 72^{o}C lasting 7 mins and a final hold at 4^{o}C using Pettier based Thermo cycler. All PCR products were checked for bands on 1.5% agarose gels. PCR product was mixed with 2 µl of loading dye (Fermentas) and introduced into the wells in the gel. A standard DNA ladder (Fermentas-10,000 bp) was also loaded in the gel well.The amplification result was detected on 1.5% agarose gel electrophoresis stained with 10mg/ml of ethidium bromide and visualised by ethidium bromide-UV fluorescence to determine the size of the amplified bands. Gel pictures were taken using the gel documentation system (Alphamager^{TM}).

2.12 Sequencing and Phylogenetic Analysis

Sequencing of the PCR product was done using the PROMEGA Wizard SV Gel and PCR purification kit. Forward and reverse sequences of the 16S rRNA regions obtained were aligned using BioEdit software. Thereafter, the homologous sequences were compared using the basic local alignment search tool (BLAST) on the National Center for Biotechnology Information (NCBI) website to obtain the nearest identical organisms based on the percentage similarity. Taxanomic identification was achieved by comparing the obtained nucleotide sequences for similarity with reference strains by a BLAST search within the NCBI gene bank. Phylogenetic analysis was carried out using CLUSTAL W software. The neighbour-joining (NJ) method was used to infer the evolutionary history of the identified organisms and the bootstrap consensus tree was inferred from a number of replicates. The positions in the final dataset were used as the out group.

3. RESULTS AND DISCUSSION

3.1 Identification of Ore

The iron ore from Agbaja was identified through X-ray crystallographic diagram of the X-ray crystallography (Fig 1) . Analysis of this revealed that iron from the ore had the highest concentration value of 25.10 mg/g as compared to the other ganged metals (zinc, copper and lead) with concentration values of 1.3903, 0.0940 and 0.2813 mg/g respectively. While nickel concentration was below detection level.

3.2 Molecular/Phylogenetic Studies

Fig. 4-5 are results of various molecular determinations from two of the organisms (*Acidithiobacillus* and *Leptospirillum* species) whose DNA were found to be of high quality. The extracted genomic DNA from the two organisms clearly showed that they had molecular weight above 1kbp which were subsequently subjected to PCR with very conspicuous bands. Sequence and phylogenetic analysis further confirmed that these strains isolated are members of *Acidithiobacillus and Leptospirillum species.*

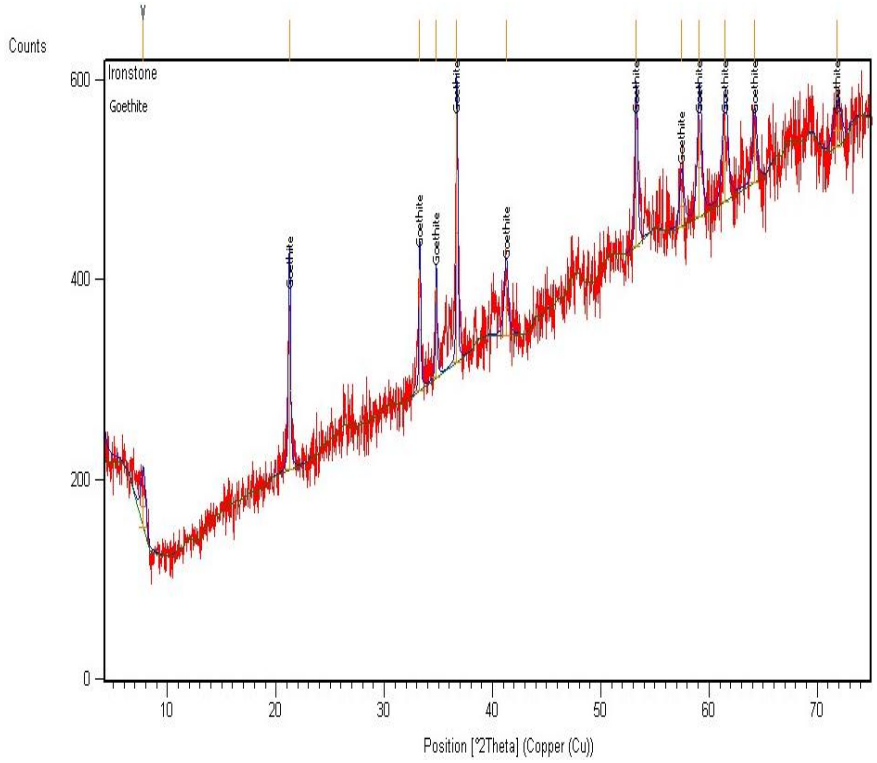

Fig. 1. Crystallographic diagram of Agbaja ironstone

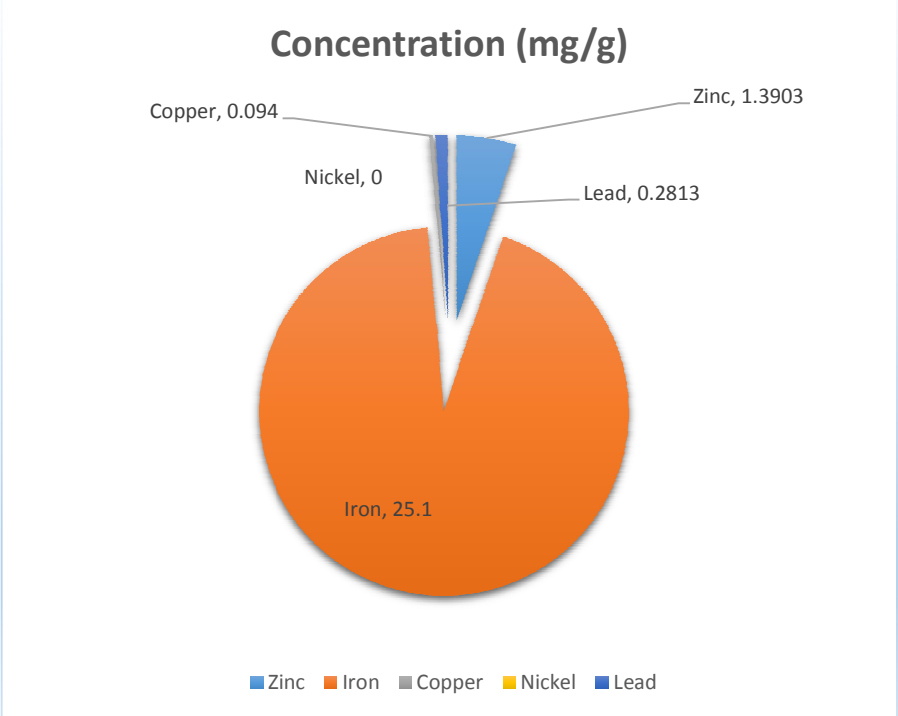

Fig. 2. Concentrations of different ganged metals in Agbaja ironstone

Table 1. Description of Agbaja ironstone from XRD crystallography

Date: 10/20/2014 Time: 2:05:39 PM File: Ironstone User: Administrator

Name and formula

Reference code:	00-029-0713
Mineral name:	Goethite
Compound name:	Iron Oxide Hydroxide
PDF index name:	Iron Oxide Hydroxide
Empirical formula:	$FeHO_2$
Chemical formula:	$Fe_{+3}O(OH)$

Crystallographic parameters

Crystal system:	Orthorhombic
Space group:	Pbnm
Space group number:	62
a (Å):	4.6080
b (Å):	9.9560
c (Å):	3.0215
Alpha (°):	90.0000
Beta (°):	90.0000
Gamma (°):	90.0000
Measured density (g/cm^3):	4.00
Volume of cell (10^6 pm^3):	138.62
Z:	4.00
RIR:	-

Subfiles and Quality

Subfiles:	Common Phase Educational pattern Forensic Inorganic Mineral Pigment/Dye
Quality:	Indexed (I)

Comments

Color:	Dark brown
Creation Date:	1/1/1970
Modification Date:	1/24/2009
Additional Patterns:	See PDF 01-074-2195, 01-081-0462, 01-081-0463 and 01-081-0464. To replace 00-017-0536
Analysis:	Chemical analysis (wt.%): "Si O2" 1.84, "Fe2 O3" 86.30, "H2 O" (<105C) 10.79, "H2 O" (<105C) 0.86, and minor MgO, CaO, "C O2" and organic C. Color: Dark brown. General Comments: Lead nitrate used as internal standard (a=7.8568). Dx for "Fe3.88 Six (O H) 4.31 O3.69" (x </= 0.012), formula from chemical analysis with impurities deducted. Opaque Optical Data: Opaque mineral optical data on specimen from Restornel, Cornwall, England, UK: R1Ro=17.5, RR2Re=15.6, Disp.=16, VHN=667 (mean at 100, 200, 300), Color values=1 .291, .296, 17.5, 2 .294, .299, 15.5, Ref.: IMA Commission on Ore Microscopy QDF. Sample Source or Locality: Specimen from Hindlow quarry, Derbyshire, England, UK (E35891). Unit Cell Data Source: Powder Diffraction.

1 Of 3

Table 2. Morphological and biochemical characteristics of the isolated organisms

Isolates	Form	Colour	Opacity	Edges	Elevation	Cellular Morphology	Gram Stain	Oxidase	Catalase	NaCl	Citrate	Sucrose	Glucose	Fructose	Urease	Tentative Microorganism identified
1	Circular	White	Opaque	Entire	Flat	Rod	-	-	+	-	+	A	A	A	+	*Acidithiobacillus sp*
2	Circular	Cream	Opaque	Entire	Flat	Rod	-	-	-	-	+	+	+	A	-	*Leptospir-illum sp*

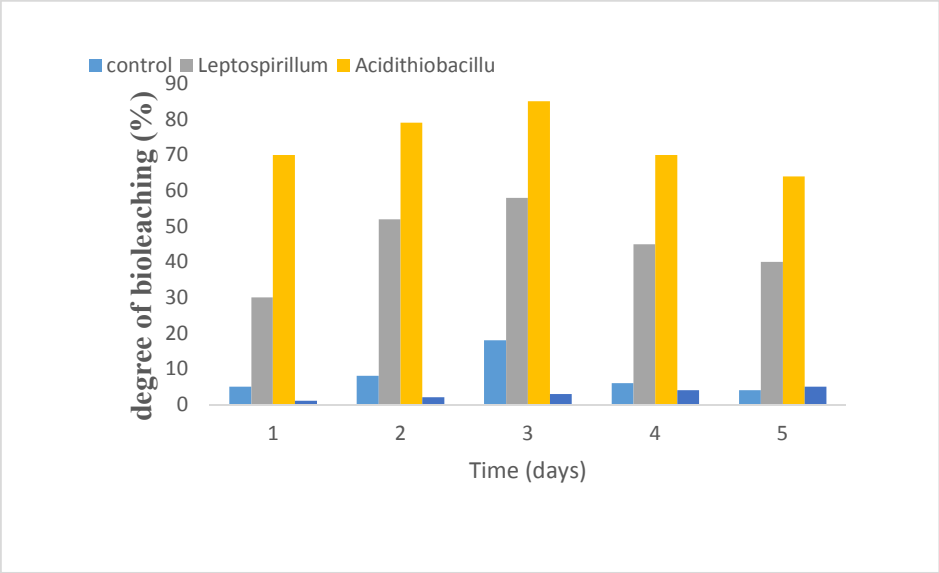

Fig. 3. Degree of bioleaching of the different bacterial species

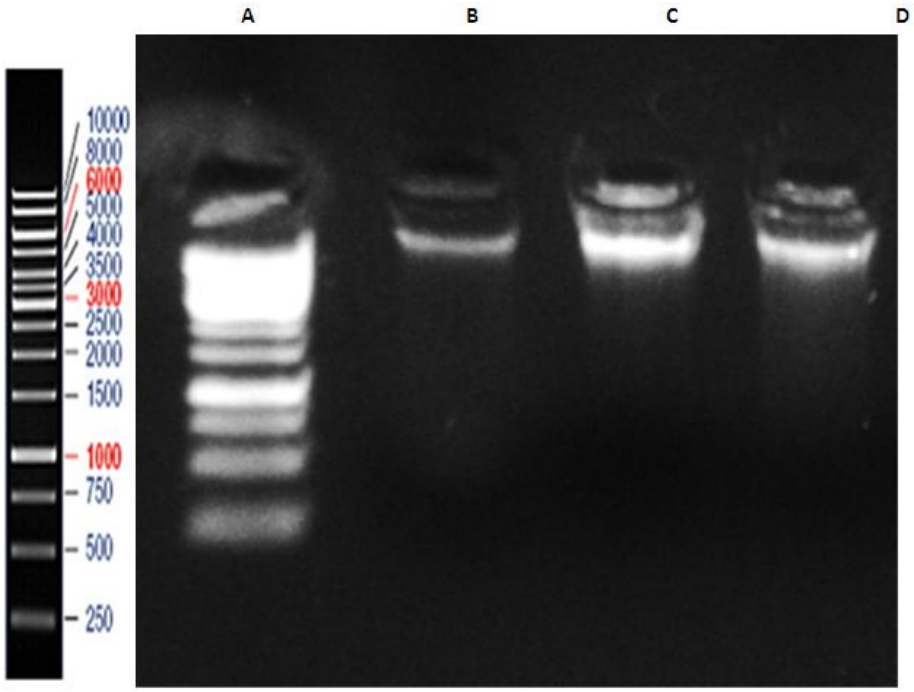

Fig. 4. Gel electrophoresis micrograph of genomic DNA from extracted isolates of Agbaja ironstone

A- Standard marker Lane
B- Lane Corresponding to Acidithiobacillus species 1 genomic DNA at 3.3 kbp
C- Lane Corresponding to Leptospirillum species genomic DNA at 3.2 kbp
D- Lane Corresponding to Acidithiobacillus species 2 genomic DNA at 3.5 kbp

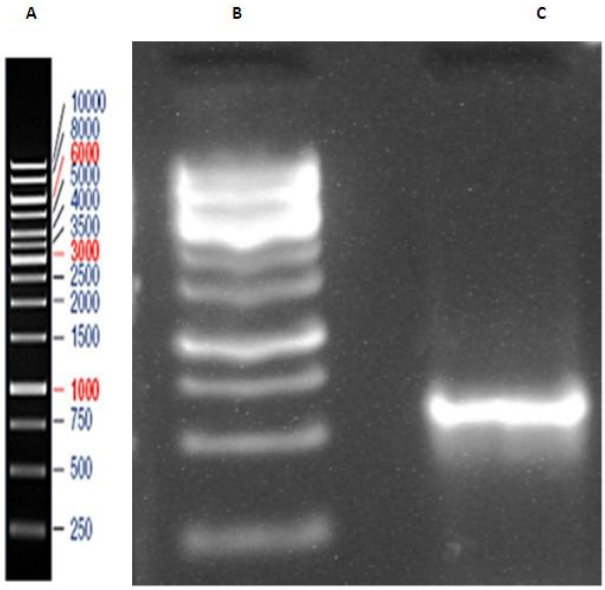

Fig. 5. PCR amplification of *Leptospirillum Specie*
A-Standard Marker Lane
B- Amplified DNA corresponding to 1.3kbp
C-

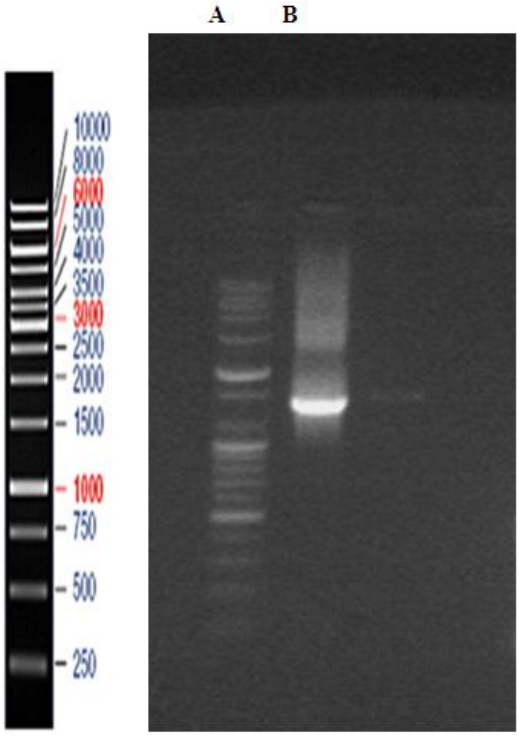

Fig. 6. PCR amplification of *Acidithiobacillus* species
A- Standard Marker Lane
B- Amplified DNA corresponding to 1.5kb

3.3 Blast

***Leptospirillum* species Sequence alignment with the NCBI gene bank database sequences**

CAFJWUK1013
Query ID 84273
Description None
Molecule type nucleic acid
Query Length 38
Database Name Representative genomesref_prok_rep_genomes
Description
Program BLASTN 2.6.1+ Citation

Graphic Summary

Distribution of the top 94 Blast Hits on 35 subject sequences

Descriptions

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus bogoriensis ATCC BAA-922 T323DRAFT_scaffold00041.41_C, whole genome shotgun sequence	71.3	71.3	100%	8e-11	100%	NZ_JHYI01000050.1
Bacillus coahuilensis m4-4 1955101000013, whole genome shotgun sequence	71.3	71.3	100%	8e-11	100%	NZ_ABFU01000013.3
Bacillus coahuilensis m4-4 1955101000135, whole genome shotgun sequence	71.3	71.3	100%	8e-11	100%	NZ_ABFU01000135.1
Bacillus coahuilensis m4-4 1955101000134, whole genome shotgun sequence	71.3	71.3	100%	8e-11	100%	NZ_ABFU01000134.1
Bacillus coahuilensis m4-4 1955101000131, whole genome shotgun sequence	71.3	71.3	100%	8e-11	100%	NZ_ABFU01000131.1
Bacillus azotoformans LMG 9581 contig133, whole genome shotgun sequence	71.3	71.3	100%	8e-11	100%	NZ_AJLR01000133.1
Bacillus horikoshii DSM 8719 Cont75, whole genome shotgun sequence	71.3	71.3	100%	8e-11	100%	NZ_LQXN01000040.1
Bacillus alcalophilus ATCC 27647 = CGMCC 1.3604 strain AV1934 contig00159, whole genome shotgun sequence	71.3	71.3	100%	8e-11	100%	NZ_ALPT02000159.1
Bacillus circulans NBRC 13626 DNA, contig: BCI01S_CON0057_0001, whole genome shotgun sequence	71.3	71.3	100%	8e-11	100%	NZ_BCVE01000056.1

71.3 bits(38) 8e-11 38/38(100%) 0/38(0%) Plus/Plus
Query 1 TAGGCGGCTGGCTCCAAAAGGTTACCCCACCGACTTCG 38
||||||||||||||||||||||||||||||||||||||
Sbjct 3256 TAGGCGGCTGGCTCCAAAAGGTTACCCCACCGACTTCG 3293

Score Expect Identities Gaps Strand
71.3 bits(38) 8e-11 38/38(100%) 0/38(0%) Plus/Minus
Query 1 TAGGCGGCTGGCTCCAAAAGGTTACCCCACCGACTTCG 38
||||||||||||||||||||||||||||||||||||||
Sbjct 735 TAGGCGGCTGGCTCCAAAAGGTTACCCCACCGACTTCG 698

Score Expect Identities Gaps Strand

71.3 bits(38) 8e-11 38/38(100%) 0/38(0%) Plus/Minus
Query 1 TAGGCGGCTGGCTCCAAAAGGTTACCCCACCGACTTCG 38
||||||||||||||||||||||||||||||||||||||
Sbjct 3590 TAGGCGGCTGGCTCCAAAAGGTTACCCCACCGACTTCG 3553

Score Expect Identities Gaps Strand
71.3 bits(38) 8e-11 38/38(100%) 0/38(0%) Plus/Plus
Query 1 TAGGCGGCTGGCTCCAAAAGGTTACCCCACCGACTTCG 38
||||||||||||||||||||||||||||||||||||||
Sbjct 1667 TAGGCGGCTGGCTCCAAAAGGTTACCCCACCGACTTCG 1704

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Query 1 TAGGCGGCTGGCTCCAAAAGGTTACCCCACCGACTTCG 38
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Sbjct 536 TAGGCGGCTGGCTCCAAAAGGTTACCCCACCGACTTCG 499

Score Expect Identities Gaps Strand
71.3 bits(38) 8e-11 38/38(100%) 0/38(0%) Plus/Plus
Query 1 TAGGCGGCTGGCTCCAAAAGGTTACCCCACCGACTTCG 38
||||||||||||||||||||||||||||||||||||||
Sbjct 82 TAGGCGGCTGGCTCCAAAAGGTTACCCCACCGACTTCG 119

Score Expect Identities Gaps Strand
71.3 bits(38) 8e-11 38/38(100%) 0/38(0%) Plus/Minus
Query 1 TAGGCGGCTGGCTCCAAAAGGTTACCCCACCGACTTCG 38
||||||||||||||||||||||||||||||||||||||
Sbjct 12107 TAGGCGGCTGGCTCCAAAAGGTTACCCCACCGACTTCG 12070

Fig. 7. Blast results of L*eptospirillum* species against NCBI gene bank database sequences

3.4 Blast

***Acidithiobacillus* species Sequence alignment analysis against the NCBI gene bank database sequences**

RID 8PPS4AP9014
Query ID 240395
Description None
Molecule type nucleic acid
Query Length 44
Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.6.0+
Distribution of the top 100 Blast Hits on 100 subject sequences

Descriptions
Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus cereus strain H61 16S ribosomal RNA gene, partial sequence	82.4	82.4	100%	3e-13	100%	KU922345.1
Bacillus oceanisediminis strain SH-63 16S ribosomal RNA gene, partial sequence	82.4	82.4	100%	3e-13	100%	KX959969.1
Bacillus sp. strain 22-8 16S ribosomal RNA gene, partial sequence	82.4	82.4	100%	3e-13	100%	KX816421.1
Bacillus sp. strain M-38 16S ribosomal RNA gene, partial sequence	82.4	82.4	100%	3e-13	100%	KX099296.1
Bacillus sp. strain M-21 16S ribosomal RNA gene, partial sequence	82.4	82.4	100%	3e-13	100%	KX099279.1
Bacillus sp. strain M-6 16S ribosomal RNA gene, partial sequence	82.4	82.4	100%	3e-13	100%	KX099265.1
Bacillus anthracis strain LZU-43 16S ribosomal RNA gene, partial sequence	82.4	82.4	100%	3e-13	100%	KT262979.1
Bacillus cereus strain RotSA 16S ribosomal RNA gene, partial sequence	82.4	82.4	100%	3e-13	100%	KT275132.1
Bacillus infantis strain HQB225 16S ribosomal RNA gene, partial sequence	82.4	82.4	100%	3e-13	100%	KT758424.1
Uncultured prokaryote clone seq_M-R39_16SR 16S ribosomal RNA gene, partial sequence	82.4	82.4	100%	3e-13	100%	KP409679.1
Bacillus sp. MSCS30 16S ribosomal RNA gene, partial sequence	82.4	82.4	100%	3e-13	100%	KJ882425.1
Bacillus cereus strain D42 16S ribosomal RNA gene, partial sequence	82.4	82.4	100%	3e-13	100%	KC441777.1
Bacillus sp. JN28 16S ribosomal RNA gene, partial sequence	82.4	82.4	100%	3e-13	100%	KC121049.1

```
Query  1     TAGCGGCTGGCTCCAAAGGTTACCCCACCGACTTCGGGTGTTAC  44
             ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1428  TAGCGGCTGGCTCCAAAGGTTACCCCACCGACTTCGGGTGTTAC  1385
```

Score	Expect	Identities	Gaps	Strand
82.4 bits(44)	3e-13	44/44(100%)	0/44(0%)	Plus/Minus

```
Query  1     TAGCGGCTGGCTCCAAAGGTTACCCCACCGACTTCGGGTGTTAC  44
             ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1433  TAGCGGCTGGCTCCAAAGGTTACCCCACCGACTTCGGGTGTTAC  1390
```

Score	Expect	Identities	Gaps	Strand
82.4 bits(44)	3e-13	44/44(100%)	0/44(0%)	Plus/Minus

```
Query  1     TAGCGGCTGGCTCCAAAGGTTACCCCACCGACTTCGGGTGTTAC  44
             ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1440  TAGCGGCTGGCTCCAAAGGTTACCCCACCGACTTCGGGTGTTAC  1397
```

Score	Expect	Identities	Gaps	Strand
82.4 bits(44)	3e-13	44/44(100%)	0/44(0%)	Plus/Minus

```
Query  1     TAGCGGCTGGCTCCAAAGGTTACCCCACCGACTTCGGGTGTTAC  44
             ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1441  TAGCGGCTGGCTCCAAAGGTTACCCCACCGACTTCGGGTGTTAC  1398
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Score	Expect	Identities	Gaps	Strand
82.4 bits(44)	3e-13	44/44(100%)	0/44(0%)	Plus/Minus

```
Query  1     TAGCGGCTGGCTCCAAAGGTTACCCCACCGACTTCGGGTGTTAC  44
```

```
                ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1438  TAGCGGCTGGCTCCAAAGGTTACCCCACCGACTTCGGGTGTTAC  1395
```

Fig. 8. Blast results of *Acidithiobacillus* species against NCBI gene bank database sequences

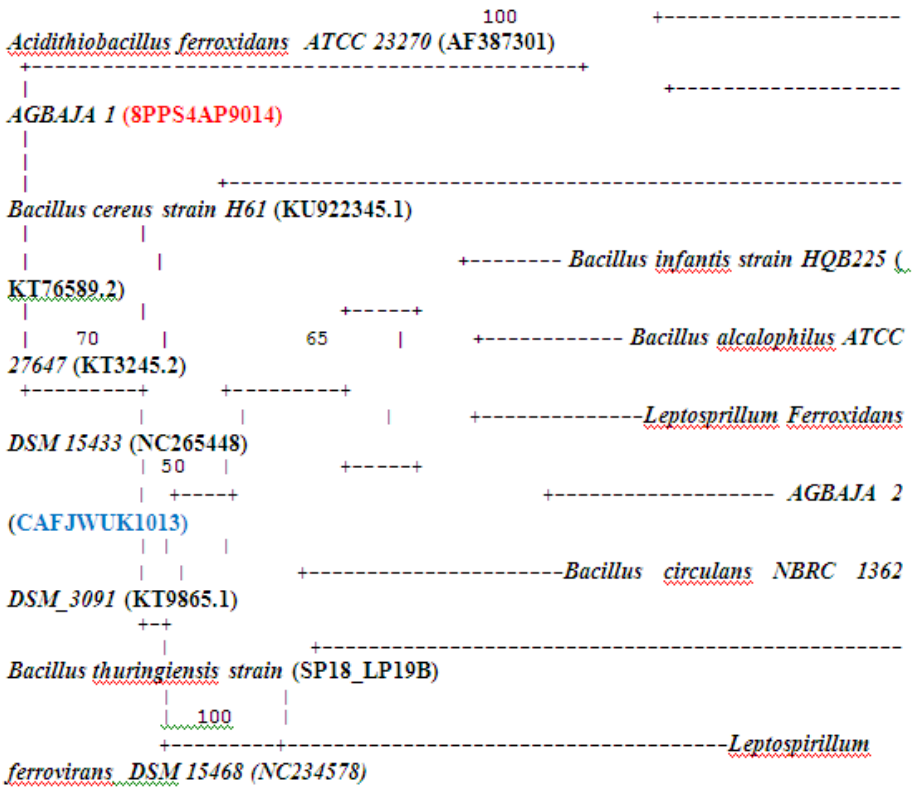

Fig. 9. Phylogenetic positions of the isolated strains from BLAST analysis

The algorithm for constructing the dendogram-neighbour-joining on the basis of comparison of 200 alternative trees. The reliability of embranchment is expressed by percentages. Scales at the left top shows evolutionary distances. Only values higher than 50% were indicated. As an outgroup the nucleotide sequences of the 16S rRNA of the type species of *Acidithiobacillus ferroxidans ATCC 23270* and *Leptospirillum ferrovirans DSM 15468* were used.

Results from X-ray diffraction (XRD) and X-ray Fluorescent (XRF) analysis of the Agbaja Iron ore reveals that iron has the highest concentration value of 25.10 mg/g as compared to the other ganged metals (Zinc, Copper, and Lead) with concentration values (1.3903 mg/g, 0.0940 mg/g and 0.2813 mg/g respectively, Nickel was below detection level. The observed concentration of iron may be due to the composition of the iron ore used for this work which has been reported in literature elsewhere to be in the region of 45-56 mg/g (Uwadiale, 1991). The Agbaja iron ore mining site is a rich reservoir of goethite ore (FeO(OH), this might have contributed to the high iron content in the raw ore. The other metals observed may be due to the occurrence of iron in most ores in form of Banded Iron formation (BIF) with some metals geologically. Also metals generally are significant natural component of all soils which might serve as nutrients for plants and microbes.

The entire degree of bioleaching from days 1 to 5 for a period of 4 weeks (Fig 3), suggest that

bioleaching increases with time and also a function of the growth condition of the bioleaching organisms. This is in conformity with Chime et al. [10] who reported that iron ore bioleaching bacteria follow the normal bacteria growth kinetics. Metals and their compound interact with microbes in various ways depending on the type of metal, organism, environment, and cellular composition of the microbes. This might have accounted for the fluctuation in the rate of bioleaching of the diverse mixed consortium observed in this work. Microbes are intimately associated with biogeochemical cycling of metals and elements as a result of immobilization depending on the mechanism involved and the micro flora of organisms [11]. Bacteria resistance mechanisms generally involve efflux or enzymatic detoxification which can lead to the release of some metallic ions from the cell, as a result of this mechanism, microbes (especially bacteria) may synthesize varieties of metals binding peptide and proteins which regulate metal ion homeostasis and can also affect toxic responses. The variations in the bioleaching rates seen in both pure cultures and mixed consortia could also be as a result of some microbial process like energy generation, nutrient acquisition, and cell adhesion and biofilm formation [12]. In addition, potentially toxic metals released from minerals as a result of physicochemical and biological processes may also affect microbial community diversity. The bioleaching of iron as seen in the different results indicated that more than 90% of iron was solubilized after 2 days of bioleaching when mixed cultures were used and reached maximum removal efficiency of approximately 100% within the same 48 hours when the whole organisms were combined together. No significant difference in iron removal was noted between mixed cultures as opposed to the single cultures with lower bioleaching rates. The leaching of iron was related to the pH, temperature, and composition of the bioleaching medium as indicated by the concomitant sharp release of iron together with the increase in incubation time and temperature in the initial period of bioleaching [13]. The control experiment could only achieve 18% of iron bioleaching.

Molecular and phylogenetic analysis based on 16S rRNA sequence homology suggested that the isolated bacterium cultured from these Agbaja iron stones exhibited close resemblance with *Acidithiobacillus ferroxidans ATCC 23270* and *Leptospirillum ferrovirans DSM 15468* (100% sequence similarity in Fig 9). Both genus are within the β-subdivision of the proteobacteria and includes five species, *R. picketii, R. insidiosa, R. mannitolilytica, R. syzygii* and *R. solanacearum* (Welch *et al.,* 2005). Their wide geographic distribution, large host range and the exceptional capacity to adapt in many different environments made them more indigenous for the rock and soil environment especially of iron mines as attested by this findings. Further, the sequence variability of rRNAs and the advent of PCR made rRNA even more accessible for sequencing providing a unique opportunity for in-depth phylogenetic analysis to highlight the breadth of diversity within various major bacterial phyla encountered in diverse environment. Annotation of the genome sequence of *A. ferrooxidans* has confirmed the known physiological capabilities of this acidophilic iron-oxidizing and iron-reducing proteo-bacterium and provided new insights into the metabolic pathways involved [14,15,2,16] These include carbon metabolism, sulfur uptake and assimilation, hydrogen metabolism, biofilm formation, nitrogen fixation and anaerobic respiration and biology of iron oxidation. Most of these studies have been carried out with strains of American Type Cell Culture (ATCC) group of bacteria, which has been recently identified as a group II iron-oxidizing *Acidithiobacillus* specie [17], although the same mechanism has been confirmed with the type strain (group I) (ATCC 23270) of *A. ferrooxidans*. Genes encoding proteins involved in ferrous iron oxidation in these strains are located on the rus operon. Four electron transport proteins are encoded on this operon: two cytochromes c (Cyc1 and Cyc2), an aa3- type cytochrome oxidase, and the low-molecular-mass copper protein rusticyanin. Hallberg et al. [18], reported that the rusticyanin in group I and group II iron-oxidizing acidithiobacilli is type A, whereas a variant of this (type B) is found in group III (*L. ferrivorans*) and group IV ironoxidizing acidithiobacilli. Groups III and IV do not appear to possess the rus operon, and iron oxidation is thought to proceed by another, as yet not fully elucidated mechanism. Interestingly, although rusticyanin has long been postulated to play a central role in ferrous iron oxidation in At. ferrooxidans-like bacteria, the absence of both isozymes (A and B) in one strain (CF27) of *L. ferrivorans* infers that it is not essential to this function in all iron-oxidizing *acidithiobacilli* [18].

The DNA extraction method employed in this present work which made use of specially designed lysis buffer was also found to have the following advantages: Good yields of high quality

genomic DNA, Circumvention of the use of liquid nitrogen for crushing of the bacterial biomass, Reduction in the extraction steps, Minimal requirement of chemicals/toxic substances and contamination by extrenous substances. A high molecular weight DNA (>1.0kb) resulted from the use of this method. The ratio of the absorbance at 260 and 280 was found to be 1.6 indicating a good purity with very little smear on the DNA. The PCR technique further ascertain the quality of the extracted DNA. The isolated DNA produced good banding patterns for *A. ferroxidans* and *Leptospirillum species* indicating its good quality. The entire DNA extraction kit was also suitable in releasing large quantities of DNA from the bacteria culture used due to the quantity of DNA gotten.

CONCLUSION

Results of all the molecular analysis carried out shows that bioleaching efficiencies of organisms from Agbaja iron ore samples could have been enhanced by proteolysis, complexation by extracted metals, Fe^{3+} binding siderophores, and general redox reactions probably due to the genetic composition of the identified organisms. It has been shown that microbes and their extracellular products can influence the mobility of metals like Cu and Fe from mining site or dump site even under low nutrient system. Results from the BLAST and phylogenetic analysis shows that the level of similarity of the nucleotide sequences of type strains of *Acidithiobacillus ferroxidans ATCC 23270* and *Leptospirillum ferrovirans DSM 15468* showed more than 97% relationships to the isolated strains in this work. We can therefore infer that the strains of AGBAJA1 and AGBAJA 2 may be novel species of the genus *Acidithiobacillus* and *Leptospirillum* respectively.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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