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ORIGINAL PAPER

Bioleaching of copper- and zinc-bearing ore using consortia of indigenous iron-oxidizing bacteria

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Abstract

Indigenous iron-oxidizing bacteria were isolated on modified selective 9KFe^{2+} medium from Baiyin copper mine stope, China. Three distinct acidophilic bacteria were isolated and identified by analyzing the sequences of 16S rRNA gene. Based on published sequences of 16S rRNA gene in the GenBank, a phylogenetic tree was constructed. The sequence of isolate WG101 showed 99% homology with *Acidithiobacillus ferrooxidans* strain AS2. Isolate WG102 exhibited 98% similarity with *Leptospirillum ferriphilum* strain YSK. Similarly, isolate WG103 showed 98% similarity with *Leptospirillum ferrooxidans* strain L15. Furthermore, the biotechnological potential of these isolates in consortia form was evaluated to recover copper and zinc from their ore. Under optimized conditions, 77.68 ± 3.55% of copper and 70.58 ± 3.77% of zinc were dissolved. During the bioleaching process, analytical study of pH and oxidation–reduction potential fluctuations were monitored that reflected efficient activity of the bacterial consortia. The FTIR analysis confirmed the variation in bands after treatment with consortia. The impact of consortia on iron speciation within bioleached ore was analyzed using Mössbauer spectroscopy and clear changes in iron speciation was reported. The use of indigenous bacterial consortia is more efficient compared to pure inoculum. This study provided the basic essential conditions for further upscaling bioleaching application for metal extraction.

Keywords Bioleaching · Iron-oxidizing bacteria · *Acidithiobacillus ferrooxidans* · Mössbauer spectroscopy

Introduction

In human civilization, metal extraction has been an essential activity since Bronze and Iron times. Depletion of high-grade ore resources due to global rise in human population and industrial development has increased the demands of metals. Over the previous several years, extraction of metals from low- and lean-grade ores using microorganisms has been developed into an effective and growing area in biotechnology (Panda et al. 2015). These microorganisms catalyze the metals recovery by dissolution of metals present in low-grade sulfide minerals through bioleaching technique or dissolve sulfide minerals to unlock the associated metals within refractory ores (biooxidation) such as gold that would be finally extracted through conventional methods (Johnson 2013). Bioleaching has been successfully applied for copper extraction from secondary copper sulfide ores. Today, about 20–25% of copper production is achieved through bio-hydrometallurgical techniques (Panda et al. 2014). Reduction of high-grade ore resources is not only concern for mining industry but the concurrent increase in low-grade ores leads to numerous environmental issues and

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occupies additional land area because of higher dump activities (Panda et al. 2015).

Several selected bacteria are gaining momentum in bioleaching to extract metals from their respective ores in more economic and environmentally friendly way (Zeng et al. 2015; Mishra et al. 2016; Yang et al. 2016). Numerous acidophilic chemolithotrophic bacteria have been reported and characterized. Iron- and sulfur-oxidizing bacteria *Acidithiobacillus ferrooxidans* that efficiently acts for dissolution of metals from their respective ores through a biochemical mechanism that is now well explained (Sand et al. 2001; Osorio et al. 2013). In addition, the use of sulfur-oxidizing *Acidithiobacillus thiooxidans* along with iron-oxidizing bacteria established a remarkably effective consortium for extraction of heavy metals from their ores and industrial wastes (Baba et al. 2011; Panda et al. 2013b). Such consortia of iron- and sulfur-oxidizing acidophiles are difficult to contaminate by unwanted microorganisms that make consortia industrially important for novel applications in many sectors (Mishra et al. 2016; Zhu et al. 2011). Bioleaching studies of several minerals have exhibited satisfactory recovery of metals using acidophilic bacteria. However, some difficulties such as higher sensitivity of bacterial cell wall towards pulp density, lower ability of metal tolerance, and higher energy requirement to maintain microbial populations have limited its applications to upscale. Nowadays, attention has been given to use of acidophilic consortia in bioleaching processes (Panda et al. 2012, 2013a). Recently, consortia of acidophilic bacteria such as *At. ferrooxidans, At. thiooxidans*, and *Leptospirillum ferrooxidans* have shown auspicious results for copper extraction from low-grade ores and a favored consortium for large-scale heap bioleaching process (Panda et al. 2012, 2015). It is strongly believed that the indigenous microorganisms obtained from the same site would be more efficient to recover metals from the ores as indigenous bacteria are more compatible with the mineralogy of the rocks. In view of the above facts, culturable diversity of acidophilic iron-oxidizing bacteria was studied from Baiyin copper mine stope, China. Owing to the reputation of these commercially vital bacteria, further, the biotechnological potential of these indigenous bacteria in consortia form was evaluated to recover copper and zinc from the ore body. Physico-chemical parameters have been optimized for efficient metal extraction and appropriate conditions have been established for upscaling the study.

Materials and methods

The entire chemicals and reagents consumed in current research were of analytical grade and purchased from Sigma-Aldrich Chemical Co and Merck.

Isolation of iron-oxidizing bacteria

Indigenous iron-oxidizing bacteria were isolated by inoculating acid mine drainage (AMD) water collected from Baiyin copper mine stope, China in a highly selective 9KFe^{2+} medium with pH 1.5 (Silverman and Lundgren 1959). The composition of the 9KFe^{2+} medium (g/L): [$(NH_4)_2SO_4$, 3.0; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.1 and $Ca(NO_3)_2$, 0.012] separately autoclaved in distilled water (900 mL) having pH 2.0. Hydrated ferrous sulfate ($FeSO_4 \cdot 7H_2O$) of 44.4 g was dissolved in 100 mL of distilled water having pH 1.5 and sterilized by filtration (0.22 µm Millipore GVWP filters) and mixed with the basal salt solution and final pH of 1.5 was adjusted with diluted sulfuric acid. AMD water of 5 mL was inoculated in 95 mL of 9KFe^{2+} media in 250-mL Erlenmeyer flasks and incubated at 150 rpm, 30 ± 0.5 °C for 15 days and control was run in parallel. The media color was regularly checked for growth confirmation of iron oxidizers. Culture broth of 2 mL was re-inoculated to fresh 9KFe^{2+} medium and re-incubated. This practice was repeated three times and bacteria were harvested through centrifugation at 14000 rpm for 20 min at 4 ± 0.5 °C. The pellet containing cells was resuspended in sterile acidified water (pH 1.5) and put into sterile separating funnel and incubated overnight at 4 ± 0.5 °C. All the suspended ferric iron particles were settled down and the milky supernatant containing bacteria was collected and again centrifuged at 14000 rpm for 20 min at 4 ± 0.5 °C. The obtained pellet was washed two times with sterile acidified water. Cells collected at pellet were suspended into sterile acidified water and spread 100 µL on solid 9KFe^{2+} medium of final pH 2.5 having agarose (0.5% w/v separately sterilized in 100 mL distilled water) as a solidifying agent and incubated at 30 ± 0.5 °C for 20 days. Plates were regularly checked for growth of bacteria, colonies were sub-cultured to purify and preserved in 20% glycerol at − 80 ± 0.5 °C.

DNA extraction and phylogenetic analysis

In the late exponential phase, the bacterial cells were harvested through centrifugation and commercially available DNA isolation kit was used according to manufacturer instruction for DNA extraction (ThermoFisher Scientific A29790). The extracted DNA was resuspended in 70 µL TE buffer mixed with RNase and its quantity and quality was assessed on agarose gel 0.8% (w/v) and by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) and stored at 4 ± 0.5 °C for subsequent analysis.

For the identification of bacterial isolates, sequencing of 16S rRNA gene was performed. Almost full length of

the gene was amplified from extracted DNA using 27F' (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R′ (5-TACGYTACCTTGTTACGACTT-3) bacterial primers (Hassanshahian and Ghoebani 2018). PCR reaction mixture of 20 µL consisted of DNA sample 1 µL, 2 µL of deoxynucleotide triphosphate (dNTP), PCR buffer 2, 2 µL each reverse and forward primer, ex taq DNA polymerase 0.5 µL (Takara Shuzo, Otsu) and 10.5 µL PCR water. First, the PCR reaction mixture was incubated at 96 °C for 4 min, and 30 amplification cycles were completed at 94 °C for 45 s, 55 °C for 60 s, and 72 °C for 60 s. Further, the reaction was incubated for 7 min at 72 °C. *E. coli* genomic DNA was included in PCR as a positive control along with negative control. Purified PCR product was obtained using Montage PCR clean up kit (Millipore) to eliminate unincorporated dNTPs and primers. This purified PCR product was sequenced using, 518F' (5′-CCA GCAGCCGCGGTAATACG-3′) and 800R' (5′-TACCAG GGTATCTAATCC-3′). Sequencing was carried out at Beijing Genomics Institute (BGI), People's Republic of China.

The obtained sequences were checked for chimeras using the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) and compared with the deposited sequences of 16S rRNA gene in public database GenBank using BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/). Closely related sequences to our unknown sequences gained from the GenBank database were aligned with unknown sequence using BioEdit 6.0. A phylogenetic tree was constructed via maximum Likelihood method with robustness of 1000 bootstrap value in MEGA 6.0 (Tamura and Nei 1993). The obtained sequences of our isolates were submitted to GenBank and accession numbers were assigned.

Ore sample

Ore sample was collected from the same site of Baiyin copper mine (36°39′3″N, 104°13′30″E, 1755 m) closed to AMD. Physico-chemical and microbiological distribution of the site has been discussed in our previous study (Sajjad et al. 2018). The chemical composition of the ore sample was found; Cu 0.5%, Fe 1.35%, and Zn 0.05% using ICP-OES (PE Optima 8000). Mineralogical analysis of the sample was quantitatively analyzed through X-ray diffraction (XRD) and results exhibited the presence of 75.3% quarts (SiO_2), 2% pyrite (FeS_2), 1.0% siderite ($FeCO_3$), 0.8% barite ($BaSO_4$), and 17% total clay. Chalcopyrite ($CuFeS_2$), sphalerite ((Zn,Fe)S), goethite (α-FeO(OH)), and galena (PbS) were present in smaller concentrations. The ore sample was ground to 5 mm size with ASTM sieves in a closed circuit and bioleaching process was employed using consortia of indigenous iron-oxidizing bacterial strains for copper and zinc dissolution.

Bacteria and culture conditions

Consortia of indigenous iron-oxidizing bacteria were used as an inoculum in the present study of bioleaching. For the bacterial consortia, the 9KFe^{2+} medium was used for their standard growth and execution of bioleaching process. Repeated sub-culturing was done in the 9KFe^{2+} medium at 30 ± 0.5 °C and 150 rpm for the activation of bacteria. Equal concentration of each isolate was mixed and the final average bacterial concentration used for bioleaching was counted 3.0×10^9 cells/mL using hemocytometer (Olympus CX31, Japan).

Bioleaching study and optimization of parameters

All the bioleaching studies were performed in 250-mL Erlenmeyer flasks in shaking incubator at 30 ± 0.5 °C, 150 rpm using 10 mL of bacterial consortia in 90 mL of sterilized 9KFe^{2+} medium. During the process, variation in pH, bacterial concentration, oxidation reduction potential (ORP) and the dissolution of metals were regularly recorded. Effect of different parameters was checked on bioleaching and experiments were performed at different pH values (1.0, 1.25, 1.5, 1.75, 2.0), pulp density (5, 10, 15, and 20%), and temperature (20, 25, 30, 35, and 40 °C) to optimize. Control set under similar conditions without bacterial inoculum was run in parallel. Water lost due to evaporation was periodically supplemented by adding the 9KFe^{2+} medium.

Analysis

Two milliliter samples were taken in sterilized condition at regular interval of time after 2 days for 10 days and concentrations of dissolved metals were analyzed using ICP-OES (America Baird Co. PS-6). The pH of the bioleaching liquor was regularly monitored by pH Meter (Model 361 provided with combined glass electrode) and the ORP of bioleaching system was measured using Pt electrode with reference to an Ag/AgCl electrode (3.0 M KCl) (BPP-922). Within inoculum, the bacterial counts were regularly carried out using hemocytometer. The percentage concentrations of dissolved metals were calculated using the formula:

$$\text{Metal extraction } (\%) = \frac{\text{Metal content in the solution}}{\text{Metal content in the ore sample}} \times 100.$$

Further, the bioleaching effect on the ore sample in the presence of iron-oxidizing bacterial consortia was studied by utilizing very sensitive instrumental analysis of Fourier transform infrared spectroscopy (FTIR) for comparison the spectral patterns.

Iron speciation

Iron speciation of the ore sample was analyzed using Mössbauer spectroscopy. About 250 mg of finely powdered ore sample after bioleaching was gently pressed into a brass sample holder (16 mm in diameter, 1 mm thick) for ^{57}Fe Mössbauer spectroscopy analysis. The sample was freeze dried and crushed. The brass sample holder was closed with an iron-free plastic cap at both ends. The Mössbauer spectra were obtained with an Austin Science S-600 Mössbauer spectrometer using a γ-ray source of 1.11 GBq ^{57}Co/Rh at a constant temperature of 19.85 °C. The obtained spectra were fitted to Lorentzian line shapes using standard line-shape fitting routines (Zheng et al. 2010). Peak intensity and half-width (HW) of each quadruple doublet were restricted to be equal. Isomer shifts (IS) were expressed about the centroid of the spectrum of metallic iron foil.

Statistical analysis

Data were analyzed using Microsoft Excel sheets and GraphPad, version 5.00 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com). Metals' concentrations were analyzed in triplicate and described as a mean value ± SD.

Results

Isolation and identification of indigenous acidophiles

The color of the 9KFe^{2+} medium was changed from pale green to deep reddish that shows the growth of indigenous iron-oxidizing bacteria. Colonies of iron-oxidizing bacteria were obtained on agarose-gelled solid plates of the 9KFe^{2+} medium when the enriched liquid sample was spread onto them. The bacterial colonies were differentiated based on morphology and size. The colonies were stained with ferric iron precipitates. Three different iron-oxidizing bacterial isolates were reported after 20 days of incubation. These strains were confirmed by phylogenetic analysis of 16S rRNA gene sequences (Fig. 1). The 16S rRNA gene sequence of iron-oxidizing bacterial isolate WG101 showed

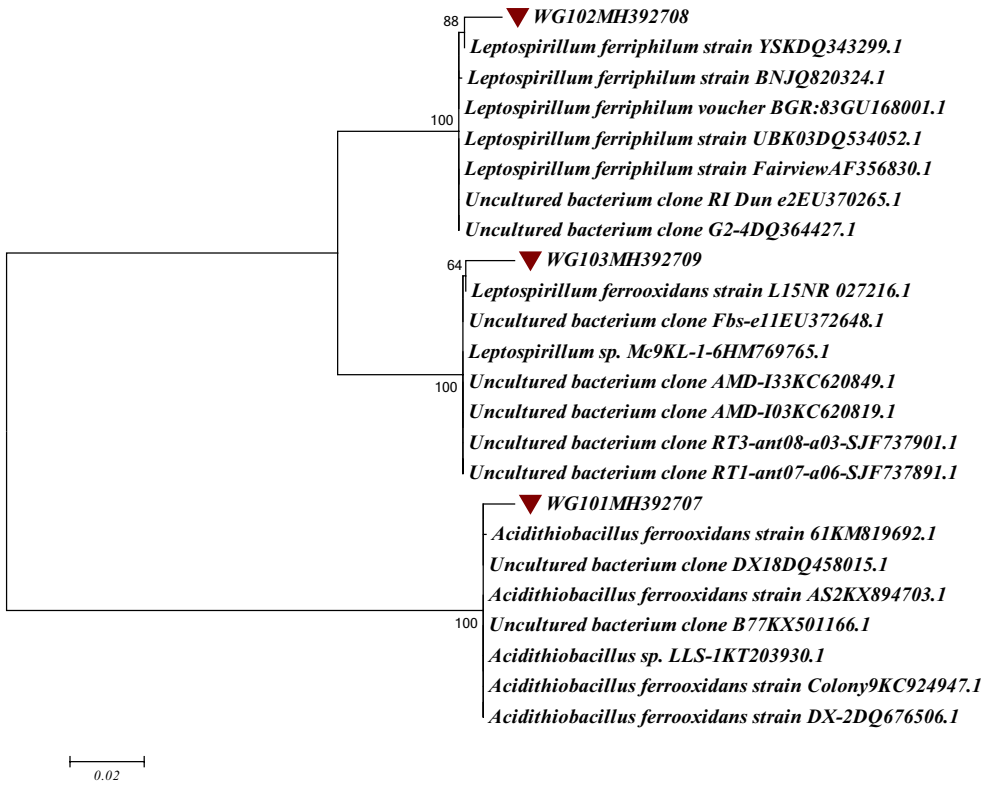

Fig. 1 Molecular phylogenetic analysis of indigenous iron-oxidizing bacteria by maximum likelihood method based on homologues sequences obtained from GenBank database. The tree with the highest log likelihood (−3431.6230) is shown. The analysis involved 24 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). The scale bar represents one nucleotide substitution per 100 nucleotides of 16S rRNA sequence. All positions containing gaps and missing data were eliminated. There were a total of 1303 positions in the final dataset. Bootstrap values of 1000 are shown

99% homology with *Acidithiobacillus ferrooxidans* strain AS2 in the GenBank. Isolate WG102 exhibited 98% similarity with *Leptospirillum ferriphilum* strain YSK isolated from acid mine drainage. Similarly, isolate WG103 was 98% similar with *Leptospirillum ferrooxidans* strain L15 reported from natural acidic environment. This shows that the site studied here is rich of iron-oxidizing bacterial diversity. The 16S rRNA gene sequences of all the three isolates were submitted to GenBank (NCBI) and accession numbers were assigned (WG101 = MH392707; WG102 = MH392708; WG103 = MH392709).

Effect of pH on bioleaching

Effect of initial medium pH on copper and zinc dissolution by consortia of indigenous iron-oxidizing bacteria was studied (Fig. 2a, b). Both copper (69.24 ± 4.34%) and zinc (65.24 ± 3.76%) dissolutions were maximum at initial pH 1.5 after 6 days of processing and further no obvious increase was noticed. The least extraction rates of copper (14.78 ± 2.7%) and zinc (17.24 ± 2.4%) were observed at pH 1.0. At higher pH of 1.75 and 2.0, an increase of metals' dissolution rate was noticed for first 6 days and then sudden decline was observed in dissolution rate. Compared to higher pH, the lower pH of 1.25 showed consistency in case of copper and a slight decline in case of zinc dissolution.

Effect of pulp density on bioleaching

The impact of pulp density on copper and zinc dissolution through bioleaching with indigenous bacterial consortia under speed 150 rpm was investigated (Fig. 2c, d). Results indicated a considerable increase in copper (66.79 ± 3.22%) and zinc (60.26 ± 3.27%) dissolutions in pulp density of 10% (w/v) till 6 days of processing and no further increase was observed. The trend showed by 5% pulp density remains constant after maximum dissolution. It is observed that the higher pulp densities of 15 and 20% initially showed increase in metals' dissolution but declined was observed later even lower than 5% pulp density. In case of copper, the declined trend was different, at 15% pulp density declined was observed after 6 days and after 4 days decline was observed in case of 20% pulp density. However, in case of zinc, the decline was observed after 6 days both in 15 and 20% of pulp densities.

Effect of temperature on bioleaching

The influence of temperature on copper and zinc dissolutions through bioleaching with indigenous bacterial consortia at initial pH of 1.5 speed 150 rpm was explored (Fig. 2e, f). Maximum copper (69.92 ± 3.0%) and zinc (64.6 ± 3.62%) dissolutions were reported at 30 °C. At lower and higher temperatures of 20 and 40 °C, the dissolution rate was very low and a decline was observed after 6 days of processing. At 25 °C, dissolutions of copper (47.34 ± 2.45%) and zinc (42.48 ± 2.34%) were higher compared at 35 °C that were 41.4 ± 2.0 and 35.57 ± 2.1%, respectively.

Bioleaching study under optimum conditions

Bioleaching of copper and zinc from the ore body during 10 days of processing using indigenous iron-oxidizing bacterial consortia under optimized conditions are studied. Copper and zinc extraction rates both in bacterial and control systems in shaking conditions were higher (Fig. 3a, b). During first 6 days, continued increase in extraction rates of both copper and zinc were observed and further there was no obvious increase noticed till 10 days of processing. At 6th day of processing, the copper and zinc recovery rates were 77.68 ± 3.55 and 70.58 ± 3.77%, respectively.

pH and oxidation–reduction potential (ORP) profiling

Figure 3c shows the pH fluctuation profiling of the bioleaching process carried out with indigenous consortia of iron-oxidizing bacteria. The pH was increased in first 2 days and then decreased for about 6 days and became constant further. Figure 3d shows ORP (mV) changes in bioleaching experiments. The redox potential of bioleaching system changed between 355 and 586 mV. Maximum metal dissolution was observed after 6 days of processing where the redox potential was higher (586 ± 6). After 6 days of bioleaching process, no abrupt change was observed in redox potential.

FTIR analysis

Further, the effect of bioleaching on ore body in the presence of indigenous iron-oxidizing bacterial consortia utilizing very sensitive instrumental analysis for the assessment of spectral patterns using FTIR is shown in Fig. 4. FTIR (Bruker Alpha II) analysis with a scanning speed of 0.2 cm/s and power less than 1 mW showed substantial alterations in the spectral pattern of bioleaching residues compared with control sample without bioleaching. The FTIR pattern of the bioleached sample subjected to bacterial treatment generated dissimilar pattern having different peak positions to the control sample.

Iron speciation

The ^{57}Fe Mössbauer spectra of controlled and leached samples measured at room temperature (RT, constant around 19.85 °C) are shown in Fig. 5. In Fig. 5a, the control sample without bacterial consortia inoculum

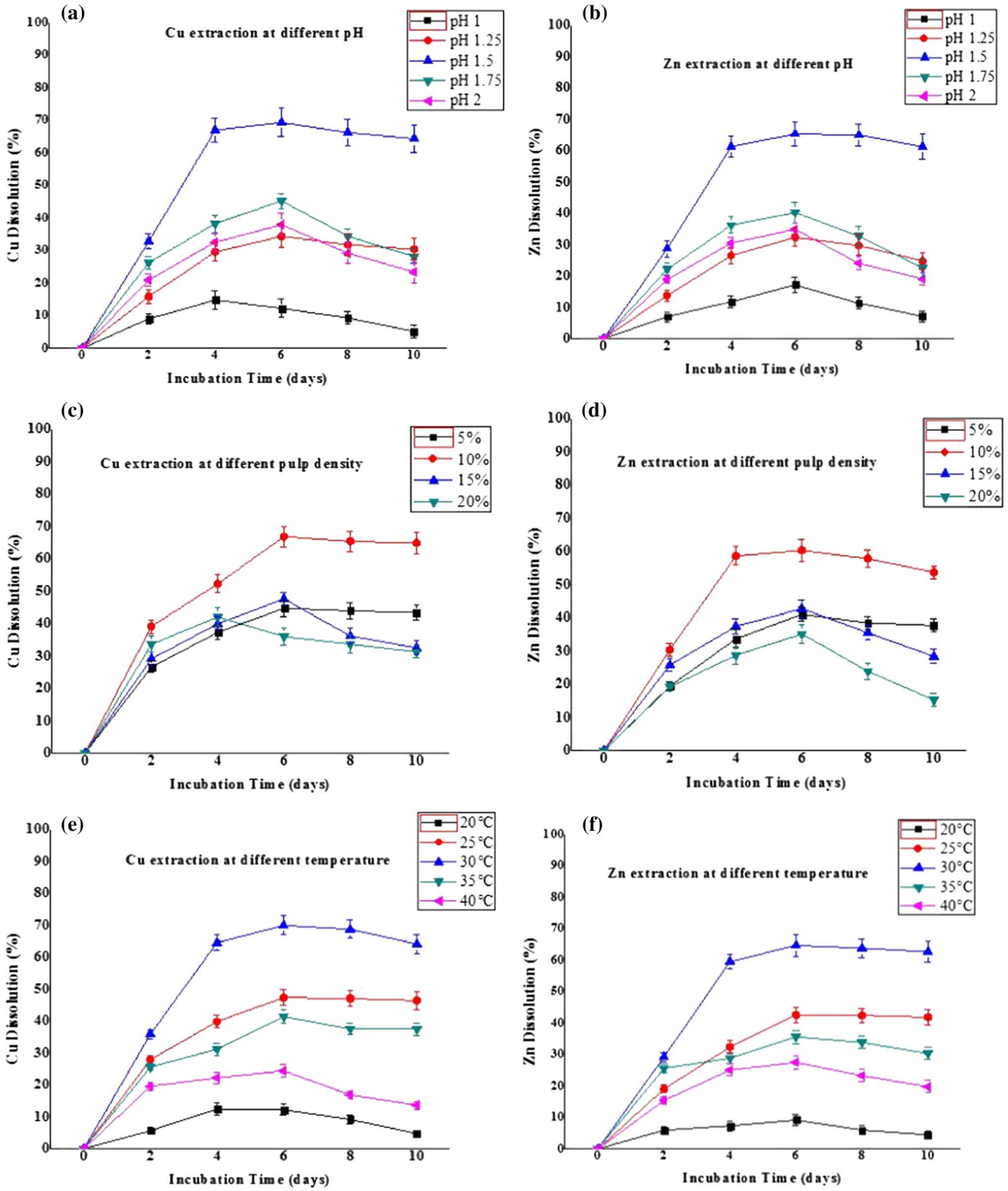

Fig. 2 Bioleaching extraction at different initial pHs **a** copper, **b** zinc; at different pulp densities **c** copper, **d** zinc; at different incubation temperatures **e** copper, **f** zinc with respect to time by the indigenous iron-oxidizing bacterial consortia. Each data point is the mean percentage for triplicate analyses (mean data ± Standard error)

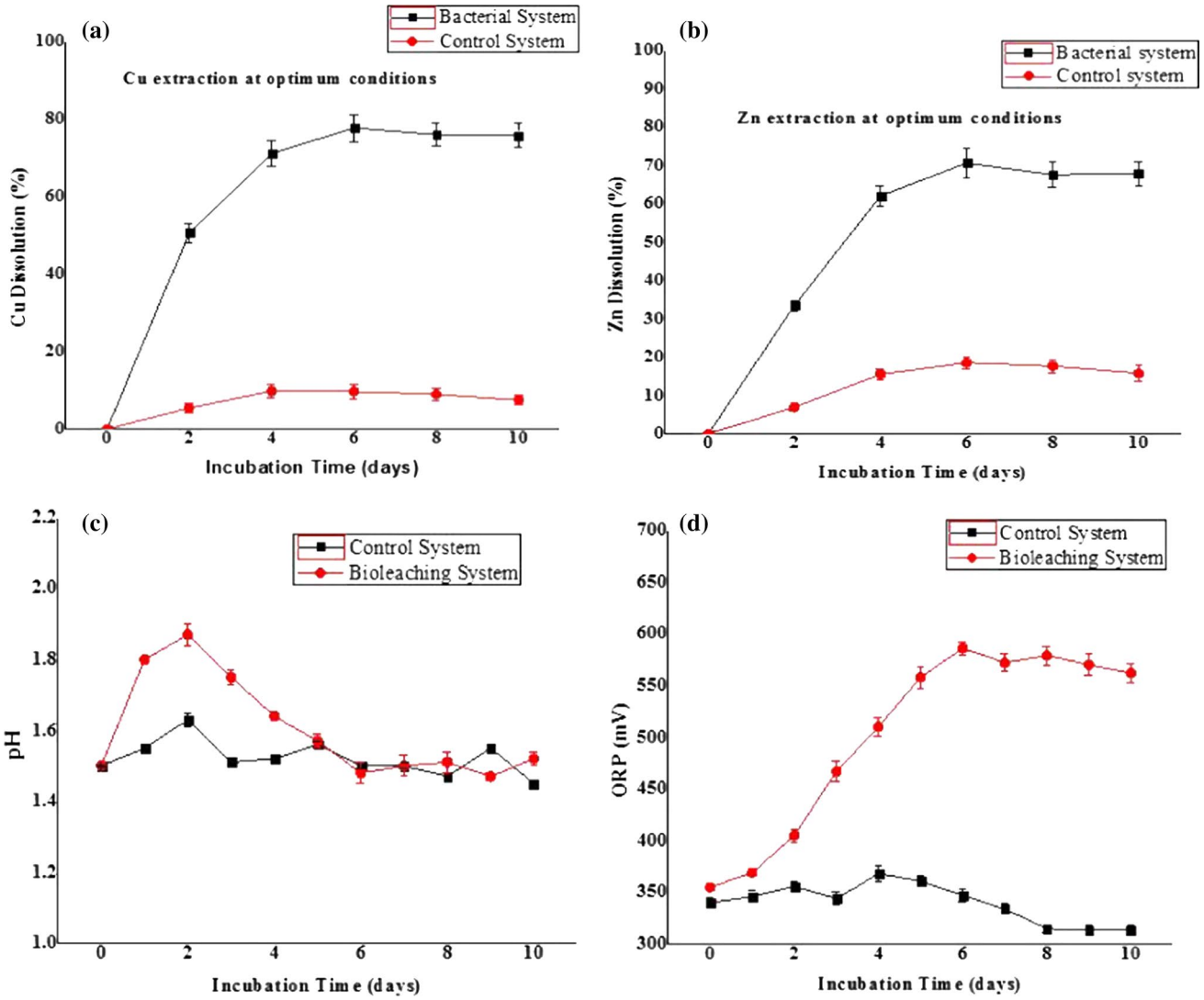

Fig. 3 Bioleaching extraction of **a** copper, **b** zinc; with respect to time at optimum conditions (Initial pH was 1.5, pulp density was 10% (w/v), inoculum density was 10%, temperature was 30 °C, and at 150 rpm) by indigenous iron-oxidizing bacterial consortia. Fluctuation in pH (**c**), and the oxidation–reduction potential (mV) (**d**), changes during 10 days of treatment. Each data point is the mean percentage for triplicate analyses (mean data ± standard error)

shows five types of sub-spectrums in which only type 4 attributed to hematite was sextet and the remaining are doublet. In case of the bioleached sample (Fig. 5b), three sub-spectrums are present in which type 3 is sextet while the remaining two are doublet. Iron species such as $FeSO_4$ and *para*-Fe^{+3} are absent in sample after bioleaching process. The curve fitting procedure for the iron components was robust, with sufficiently small Chi-squared values, indicating a reliable fitting. The corresponding iron species for sub-spectra in both samples are listed in Table 1.

Discussion

This study offers the isolation and use of indigenous iron-oxidizing bacterial consortia for copper and zinc dissolutions from the ore body of Baiyin copper mines. It is erratic that the commercial bioleaching of metal minerals has been industrialized as a vital and successful area of biotechnology; however, limited information of indigenous microbial population is available in natural processing

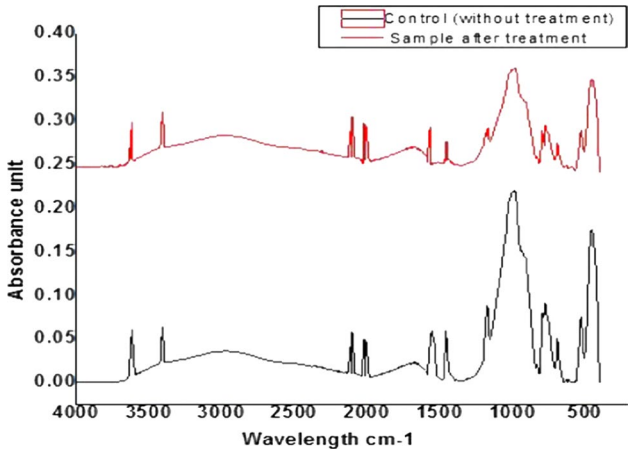

Fig. 4 FTIR spectral patterns of original host rock and leached residues after bioleaching with bacterial consortia

system. One reason is the lack of suitable and precise approaches for analysis of extremophilic microorganisms that thrive in the metal-enriched and acidic habitat. Advanced molecular techniques have been used for exploration of extremophiles, however, several potential problems associated with these techniques are (1) the extraction of DNA and other inherent biases in the quantitative analysis that results failure in detection of microorganisms altogether, (2) these techniques may entail pre-knowledge of the actual or projected indigenous microorganism present in the natural system, (3) isolation of indigenous acidophilic microorganisms on solid media. This site of copper mines was previously studied for indigenous acidophilic prokaryotes and revealed that > 95% of sequences were remained unknown and could not assigned to any known species (Sajjad et al. 2018). In fact, acidophilic microbes are poorly grow on solid media (Johnson et al. 2001), however, certain attempts were made for acidophilic bacterial

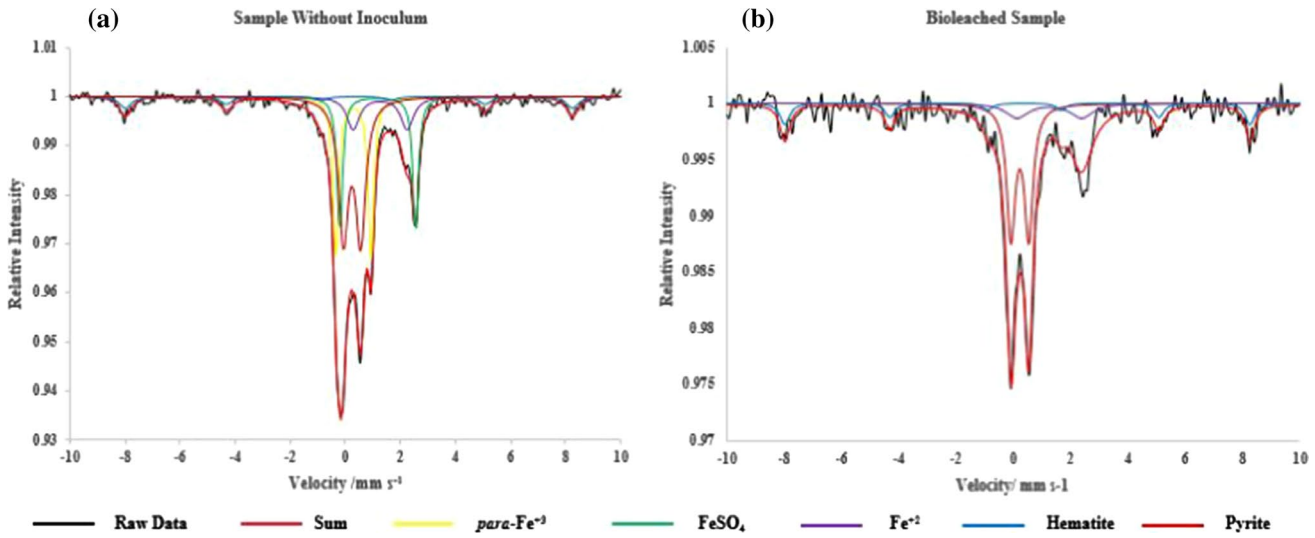

Fig. 5 Mössbauer spectra of samples **a** without inoculum, **b** with bacterial consortia measured at temperature 19.85 °C

Table 1 Mössbauer parameters of the iron species in ore samples (measured at 19.85 °C)

Samples	Species	Relative content (%)	IS (mm s^{-1})	QS (mm s^{-1})	HW (mm s^{-1})	Hi (T)
Without inoculum	*para*-Fe^{+3}	14.75	0.358 ± 0.002	1.28 ± 0.005	0.125 ± 0.01	
	$FeSO_4$	11.6	1.235 ± 0.002	2.75 ± 0.006	0.119 ± 0.02	
	Fe^{2+}	15.25	1.326 ± 0.007	1.95 ± 0.004	0.279 ± 0.04	
	Hematite	20.43	0.314 ± 0.01	− 0.25 ± 0.03	0.27 ± 0.001	50.34 ± 0.12
	Pyrite	37.95	0.31 ± 0.003	0.63 ± 0.007	0.214 ± 0.01	
With inoculum	Pyrite	39.36	0.285 ± 0.004	0.62 ± 0.001	0.179 ± 0.01	
	Fe^{2+}	29.86	1.319 ± 0.02	2.26 ± 0.05	0.468 ± 0.08	
	Hematite	30.76	0.325 ± 0.03	− 0.24 ± 0.05	0.199 ± 0.09	50.31 ± 0.17

isolation using plating techniques in recent years (Tischler et al. 2013; Auld et al. 2013; Johnson et al. 2014; Sanket et al. 2017). The plating technique used in this study exhibited effective approach for isolation of indigenous acidophilic bacteria on solid 9KFe^{2+} media used and improved by Khalid et al. (1993) with little modification in solidifying agent using agarose instead of gelrite and isolated indigenous iron-oxidizing bacteria. In the current study, culturable diversity of acidophilic iron-oxidizing bacteria was reported in copper mine stope, although it is possible that several other bacterial and archaeal species present in the sample were missed to isolate by this method. Other studies conducted showed enough isolation of acidophilic mineral-oxidizing bacteria using a special overlay plate technique (Johnson et al. 2001; Okibe et al. 2003; Auld et al. 2013). Isolates reported in the present study shows that this site is rich of iron-oxidizing acidophilic bacteria. These findings support the results of our previous study Sajjad et al. (2018) in which the presence of acidophilic bacterial species was explored and the results showed this site is rich of phylogenetically novel acidophilic bacterial diversity.

Besides providing information regarding indigenous distinct iron-oxidizing bacterial strains in the copper mine, this study led to the use of consortia of these indigenous bacteria for copper and zinc dissolution under optimized laboratory conditions. Indigenous microorganisms are more resistant towards the recalcitrant behavior of heavy metals present in leaching solutions after dissolution described by Latorre et al. (2016). Generally, mixed consortia of acidophiles are considered more effective for metals' dissolution compared to pure culture (Ciftci and Akcil 2010; Yang et al. 2013; Panda et al. 2015; Utimura et al. 2017; Deng et al. 2017) because consortium has higher capability to resist copper, zinc, arsenic and chloride ions compared to pure culture. In this study, consortia of bacterial strains were found very effective in copper and zinc dissolutions under optimized conditions. Compared to pure bacteria, the mixed inoculum shows more pronounced attachment and approximately 78.5% of consortia remained in the column either attached to the ore surface or in stagnant solution (Petersen and Dixon 2007).

Bioleaching of metals involves microorganisms, therefore, highly affected by physico-chemical factors, which in turn affect the metal extraction yield. The pH of leaching solution is an important factor in bioleaching process that controls the bacterial growth (Anders and Colin 1995). The bacterial metal dissolution was significantly different in different initial pH values of medium (Fig. 2a, b). At initial pH of 1.5, maximum metal dissolution was obtained after 6 days of processing. This maximum dissolution after 6 days is due to the use of indigenous bacterial consortia that are more compatible to the mineralogical nature of the ore. Our results showed consistency with the findings of Bhatti et al. (1993); Deveci et al. (2004); Olubambi et al. (2008). Fluctuation in initial pH of medium shows variation in dissolution rate, it is because of the inhibitory effect of pH on bacterial metabolism. Our results are in line with the study conducted by Peng et al. (2016), which revealed the pH fluctuation imposed higher impact on free cells. While at higher pH, decrease in dissolution rate was observed after 6 days of processing. The reason might be in higher pH precipitation was the predominant factor that leads to lower final dissolution rate due to pH variation that is related with the precipitate amount being produced. This pH variation is due to the consumption and regeneration of protons during bioleaching process (Eqs. 1–4). During biooxidation of ferrous iron, protons are consumed and increase in pH occurs. As the ferric iron is accumulated, its hydrolysis during leaching generates protons and in turn lowers and stabilizes the pH. Our findings of Mossbauer analysis also revealed that the ferric iron species has been changed in bioleached sample that could support the hydrolysis of ferric iron. It has been proposed that the amount of ferric iron hydrolysis was pH depended and at higher pH > 2.50 its solubility decreases (Anders and Colin 1995). In addition, the pH decreases due to the sulfuric acid production by the sulfur-oxidizing bacteria shown as (Eq. 5) (Leahy and Schwarz 2009; Zhou et al. 2009).

$$4Fe^{2+} + O_2 + 4H^+ \Rightarrow 4Fe^{3+} + 2H_2O \quad (1)$$

$$Fe^{3+} + H_2O \Rightarrow Fe(OH)^{2+} + H^+ \quad (2)$$

$$Fe(OH)^{2+} + H_2O \Rightarrow Fe(OH)_2^+H^+ \quad (3)$$

$$Fe(OH)^{2+} + H_2O \Rightarrow Fe(OH)_3 + H^+ \quad (4)$$

$$2S^0 + 3O_2 + 2H_2O \Rightarrow 2SO_4^{2-} + 4H^+ \quad (5)$$

Bacterial growth and its interaction with minerals are usually higher at low pH. While H^+ is essential for iron-oxidizing bacteria for oxidation of ferrous to ferric iron (Das et al. 2005) and bacteria takes H^+ from surrounding (Apel and Dugan 1978). As the ferrous oxidation involves H^+ consumption, therefore ferric iron generation requires higher H^+ that basically depends upon pH, concentration of ferrous and its conversion to ferric iron. Low pH corresponds to high H^+ and minerals/bacterial interaction, thus increases the tendency of contact bioleaching mechanisms. Likely, low pH promotes the ferrous oxidation to ferric iron, therefore favoring the non-contact bioleaching mechanism. On the other hand, at higher pH, the copper dissolution was reported very low it might be because at higher pH instead of ferric ion, ferric sulfate precipitates; and the copper has greater attraction for precipitation, therefore, instead of ionic form in liquid, it precipitates with the precipitated iron. Similar finding was observed by Keeling et al. (2005); Olubambi

et al. (2008) where at higher pH, soluble ferric iron was depleted due to precipitation and reduction of copper dissolution occurred. Another possible reason of lower metal dissolution at higher pH is the jarosite formation and copper could preferentially precipitate with jarosite.

Pulp density is one of the vital factors for maximum dissolution of metals. In the present study, increasing the pulp density up to 10% increased metal dissolution rate. Above this concentration, the dissolution rate was decreased and clear declined was observed in the case of higher ore concentration. Our findings are strongly supported by the results of Guo et al. (2010) and Petrus et al. (2018) where the pulp density significantly influenced the metal dissolution rate. In general, higher the pulp density than optimum in the leaching medium, obvious will be the reduction in dissolution rate. Wang et al. (2014) studied the impact of pulp density on bacterial community during bioleaching using real-time PCR and the results showed that the pulp density has significant impact on planktonic cell community. Our findings are in line with the study carried out by Olubambi et al. (2008). This is due to the inhibition of bacterial growth by the higher pulp density in the medium (Nowaczyk et al. 1998; Tipre and Dave 2004). Higher the pulp density beyond the limit will increase toxicity and inhibit bacterial activity. Additionally, higher pulp density could affect the interphase between cells and ore particles that influenced the contact of bacterial cell and particle and harm the cells. Acevedo et al. (2004) reported that high pulp density reduces the growth of bacteria and enhance the ferric reduction to ferrous rather than the oxidation of ferrous. This is because the higher pulp density can reduce the oxygen availability that is crucial for bacterial growth and the inadequate oxygen became the limiting factor for metal dissolution rate. Moreover, higher pulp density can increase the galena contents that would enhance the formation of anglesite precipitate, which reduces the bacterial activity by deterring the bacterial contact with ore surface and lowering galvanic interaction effect essential for metal dissolution. Furthermore, the bacterial cells would attach to anglesite instead on mineral surface and reduces the minerals/bacterial attachment and reduces the extraction rate.

Temperature is another vital factor that influence metal dissolution rate either by physico-chemical or microbiological reason. Therefore, the study of temperature effect on bioleaching is particularly vital (Ahonen and Tuovinen 1992; Yang et al. 2013; Cameron et al. 2010). Maximum metal dissolution rate was reported on mesophilic range of temperature (30 °C). Isolation of the indigenous iron-oxidizing bacteria used in this study was carried at 30 °C. Therefore, all the three mesophilic isolates displayed better growth and oxidation activity at temperature 30 °C of leaching solution. Our findings are comparable with the study carried out by Yang et al. (2013) for copper extraction using bacterial consortia. However, metal dissolution rate was maximum in our study, it might be due to the indigenous bacterial consortia that are compatible with ore mineralogy and significantly maintained low pH and higher ORP (Fig. 3c, d) of leaching solution. The lower dissolution rate at lower and higher temperatures could be due to the growth inhibition and the enzymatic disruption, especially at higher temperature. Effect of temperature on bacterial diversity in bioleaching system was studied by Cameron et al. (2010) using molecular technique and revealed that temperature has direct impact on bacterial diversity within bioleaching system. Cell number was reduced by the temperature increase during bioleaching of low-grade sulfide ores (Shiers et al. 2017). Therefore, under optimum conditions and using indigenous bacterial consortia, the dissolution rate was maximum for copper ($77.68 \pm 3.55\%$) and zinc ($70.58 \pm 3.77\%$) having initial medium pH 1.5, pulp density of 10%, at speed 150 rpm and 30 °C after 6 days. Our findings are similar with Guo et al. (2010), they extracted maximum metals at similar conditions after 6 days processing.

Mostly, bacteria that carried out bioleaching process fulfill their energy requirement by oxidation of ferrous to ferric or sulfur compounds to sulfuric acid (Ghassa et al. 2014; Haghshenas et al. 2012; Rohwerder et al. 2003). Generally, in bioleaching system, the ORP largely exhibit ratio of Fe^{3+}/Fe^{2+} and activity of iron-oxidizing bacteria (Manafi et al. 2013, Watling 2006). Therefore, we monitored the pH fluctuation and ORP of indigenous bacterial consortia in bioleaching system for bacterial activity and oxidation potential. During pH fluctuation, minor increased in pH of bioleaching system occurred during first 2 days and further decreased for about 6 days happened that clearly indicate the maximum bacterial activity, which mostly produced sulfuric acid and further became constant. Our findings are comparable with the results of Panda et al. (2017). Panda et al. faced this situation of pH fluctuation in similar pattern. Increase of pH occurs due to several reasons such as the presence of minor carbonate minerals and consumption of H^+ during oxidation as shown in Eq. 1. The acid-consuming materials are dominant factors in bioleaching process and the pH get increased due to acid consumption by the exposure of more acid-consuming material within ore body during bioleaching. Depends upon the chemical composition of the ore, it can be incidental that the presence of acid-consuming minerals such as Mg, K, Ca, and Na contributed pH elevation and even could inhibit the overall process. Moreover, the lepidocrocite characterized a significant amount of iron mineralization that is also acid consuming. This pH fluctuation pattern is in line with the findings of Yang et al. (2013); Ghassa et al. (2014). Addition of acid is essential to cope the pH raise, especially in the system that contains lower sulfides. To control pH at suitable level is significant for bioleaching

process due to certain reasons. First, the acidophilic bacteria are active in low pH environments. Second, low pH keeps adequate amount of ferrous iron in the leaching solution as an energy source for iron-oxidizing bacteria. Furthermore, the low pH stops the formation of passivation layer over the ore surface (Fu et al. 2008). However, acid addition results higher cost in commercial bioleaching process; therefore, the use of indigenous bacterial consortia could be more effective as reported in the present study. Initial pH fluctuation in control system was observed that might be due to the H^+ consumption by gangue materials in our sample (Panda et al. 2017).

Figure 3d shows the ORP fluctuation during bioleaching experiment. Generally, systems have no sharp fluctuations without bioleaching as shown in control of this study while sharp change was observed in bioleaching system containing $FeSO_4{\cdot}7H_2O$ for 6 days and then became relatively stable further in the range of 562–579 mV. This mainly due to the balance between consumption and generation of ferric ions. This exhibits higher oxidation of ferrous to ferric ions by indigenous iron-oxidizing bacterial consortia (Eq. 1). Higher ORP in this study showed that the bacterial consortia are more capable of iron oxidation compared to pure strain studied by Ghassa et al. (2014). The control system had smooth ORP fluctuation due to the absence of bacterial ferrous oxidation. Our findings are supported by the study of Panda et al. (2017).

Furthermore, the possible impact of iron-oxidizing bacterial consortia on ore sample was analyzed through spectral pattern using FTIR. FTIR spectra exhibited significant variations in the spectral pattern of bioleached residues compared with control. This phenomenon clearly showed that the indigenous iron-oxidizing bacteria had profound effect on the ore particles for copper and zinc bioleaching. Sometimes, the changes occur in the position of peaks can attribute to jarosite formation over the ore surface that is an eminent phenomenon (Zhu et al. 2011; Panda et al. 2013a). The peaks variation identified at the range 772.84; 1001–1080 cm^{-1} are attributed to quarts that were in higher concentration in our ore sample. The same FTIR band was reported by Prasad et al. (2006); Panda et al. (2015). At ranges 515–700, 800–818.43, 1001–1003, 1080–1175 cm^{-1}, there are obvious changes in the bands that can be attributed to V2 and V3 vibrations of SO_4, vibration of FeO_6, and OH (d-OH) deformation. Our results are strongly supported by the studies conducted by Ruan et al. (2001); Ding et al. (2007) and Panda et al. (2015). The band present at 3434.53–3455.15 cm^{-1} is attributed to the stretching of water adsorbed on the surface or OH groups present in several iron oxides particularly goethite (FeOOH) (Prasad et al. 2006; Ruan et al. 2001; Panda et al. 2015). Further, goethite is one of the strongly hydrogen bonded mineral with n(OH) stretching mode around 3100–3150 cm^{-1} (Russell and Fraser 1994; Libowitzky and Rossman 1997). Variation in the band at 3610.79–3650.96 cm^{-1} and decrease in the band after leaching occurred. This hydroxyl band indicates higher dispersion of copper (van der GRIFT et al. 1989; Panda et al. 2015). Variation in bands at 1462.45–1585.32 cm^{-1} was very clear that could be attributed to the presence of carbonates (Ruan et al. 2001), originally present in our sample.

Due to the presence of bacterial consortia in bioleaching system variation in iron speciation, reported the bacterial influence on iron speciation. Mössbauer spectroscopy is highly sensitive technique for identification of iron species in solid materials and first time used for bioleaching system. As shown in Fig. 5, both control and leached systems show different iron speciation. Doublet for $FeSO_4$ is completely absent in leached sample that can be attributed to the bacterial-based oxidation by the inoculated consortia. Similarly, the absence of *para*-Fe^{3+} spectra in case of leached system might be due to the consumption of generated ferric iron in dissolution of metals from the ore and converted back to ferrous form, which is in higher concentration (29.86%) within leached system compared to control (15.25%). It is also possible that the *para*-Fe^{3+} is converted in hematite (30.76%) due to the impact of consortia, however, the result shows that all *para*-Fe^{3+} could not be converted into hematite as the relative content of hematite is lower in bioleached system with combined content of *para*-Fe^{3+} and hematite in control system. It shows that the consumption of ferric iron in bioleaching is obvious. The other possible reason could be the hydrolysis of ferric iron in the liquid phase that maintained the lower pH of the system.

In conclusion, this study presents the isolation of indigenous iron-oxidizing bacteria using modified solid media in plating technique. The agarose solidified 9KFe^{2+} medium efficiently isolated iron-oxidizing bacteria that could be important for industrial processing it exhibited the importance of cultured-based investigation of acid mine drainage. Furthermore, the obtained strains of acidophilic iron-oxidizing bacteria were used in consortia form for copper and zinc dissolutions from the ore body obtained from the same site. Bioleaching process in the presence of bacterial consortia was performed to optimized conditions for copper and zinc recoveries. Under optimized conditions, $77.68 \pm 3.55\%$ of copper and $70.58 \pm 3.77\%$ of zinc were dissolved within 6 days of processing at pH 1.5, pulp density 10%, at 150 rpm and 30 °C; purely in the presence of bacterial consortia and without any prior pre-treatment of the ore body. During bioleaching process, analytical study of pH and ORP fluctuations was monitored that reflected efficient activity of the bacteria consortia. It was also confirmed from the FTIR analysis that variations occurred in bands after treatment with bacterial consortia. The impact of bacterial consortia on

iron speciation within bioleached ore sample was analyzed using Mössbauer spectroscopy and clear changes in iron speciation was reported. The use of indigenous bacterial consortia is more efficient compared to pure inoculum. This study provided the basic essential conditions for further upscaling bioleaching application for metal extraction from their respective ores.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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