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Research article

Performance of microbial community dominated by *Bacillus* spp. in acid mine drainage remediation systems: A focus on the high removal efficiency of SO_4^{2-}, Al^{3+}, Cd^{2+}, Cu^{2+}, Mn^{2+}, Pb^{2+}, and Sr^{2+}

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ABSTRACT

A consortium of microbial community was used for the treatment of acid mine drainage wastewater laden with sulphate and heavy metals. The wastewater was treated in an anaerobic continuously stirred tank bioreactor. The microbial community activity increased the pH from 5.6 to 6.5, and improved sulphate removal up to 85% from an initial sulphate concentration of 8080 mg SO_4^{2-}/L in a continuous mode, following enrichment for 21 d. The maximum heavy metal removal percentage was observed for Cd (98%), Al (97%), Mn (95%), Pb (94%), Sr (94%) and Cu (91%). The microbial community showed synergy between strictly anaerobic and facultative *Firmicutes* sp., which were responsible for the bioreactor performance. The biochemical reaction indicated the microbial community has a wider range of substrates dominated by metallo-aminopeptidases.

1. Introduction

Industrialisation culminated in the rise of toxicant-laden wastewater which is a threat to both aquatic and terrestrial environments when disposed-off untreated, especially in most developing nations. Mining is one of such activities on the rise owing to its impact on the economy of those nations. Although, mining activities consume a considerably smaller quantity of water compared to other industrial activities, it is the topmost producer of hazardous wastewater [1]. The mining industry is a key player in the development of South Africa's economy. Nevertheless, since mining operations cannot be relocated, many neglected mine sites are the main source of most health and environmental challenges [2]. During mineral extraction, sulphide bearing minerals are exposed to the oxygenated environment causing a cycle of complex geochemical reactions that engenders acid mine drainage (AMD) [3, 4]. When discharged untreated and/or partially treated, it drains directly into freshwater bodies and leaches into the water table leading to groundwater contamination [1]. When such contaminated water is consumed, the vital ions in cells can be replaced by heavy metals which may result in carcinogenic and mutagenic effects, including diarrhea if sulphate concentration is high [5].

Currently, there are several methods used for treating AMD, including membrane-filtration, floatation, coagulation-floatation, chemical precipitation, reverse osmosis, ion-exchange, filtration and electrochemical, amongst others [6, 7]. However, due to cost implications and environmental concerns, these methods are considered unsustainable. Besides, management of high volume of resulting sludge in chemical treatment is challenging [8]. The high rate of success recorded in anaerobic technology has encouraged researchers to explore its application to the treatment of complex wastewater such as AMD [9, 10, 11]. Treatment using sulphate-reducing bacteria (SRB) is a well-known biotechnological approach in the remediation of AMD. The biotechnological treatment provides less resolubility of sulphide precipitation than hydroxide precipitation within a wide range of pH than the chemical approach [12]. SRB are a group of microorganisms that utilise sulphate as a terminal electron acceptor. They play a major role in pollutant degradation, organic transformation and sulphur cycle in the environment [13]. When a suitable carbon source is available, SRB produces bicarbonate ions and sulphide. The bicarbonate elevates the pH while dissolved metals are precipitated by the sulphide. The reactions are summed up in Eqs. (1), (2), and (3) [14]:

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$$CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2HCO_3^- \quad (1)$$

$$Me^{2+} + HS^- \rightarrow MeS \downarrow + H^+ \quad (2)$$

$$HCO_3^- + H^+ \rightarrow H_2O + CO_2 \quad (3)$$

Where *Me* represents the metal ion.

Furthermore, microbial communities from AMD play an important role in the effective treatment of wastewater [15, 16, 17, 18]. Most of these microorganisms used in AMD remediation were found to belong to the phyla; *Firmicutes, Actinobacteria, Acidobacteria, Nitrospira, Ciliophora*, as well as *Proteobacteria* which contains both facultative anaerobic and few obligate anaerobic species [19]. Different culturing techniques have been reported in the study of microbial diversity in water and wastewater [20]. The culture-independent technique offers the benefit of precise assessment and taxonomy of microorganisms in a given sample [21, 22]. Because of the ability of microorganisms to survive and replicate in harsh environment such as extreme pH, they are considered suitable for the treatment of AMD. Similarly, metal pollution is a major concern in AMD management, analysis of microbial diversity of AMD will give a clear perception of the dominant microorganisms that can be deployed in the treatment of metal-laden AMD [19, 20]. Due to the high sensitivity of bacteria towards heavy metals, application of SRB is often limited to the wastewater with low heavy metals concentration. Consequently, most reports grow the bacteria separately before using it for the treatment [23, 24]. However, the performance of SRB grown on AMD laden with high concentrations of metals such as Al^{3+}, Fe^{2+}, Mg^{2+} & Mn^{2+} is barely been reported. Therefore, the goal of this study was to identify the group of microorganisms in the heavy metal-laden AMD samples and to ascertain the effectiveness of the identified microbial group in an AMD remediation system.

2. Materials and method

2.1. Water sampling

AMD samples were obtained from a mining facility in Mpumalanga Province, South Africa and stored as described in a previous study [25]. Briefly, AMD samples were collected using standard sampling procedure, screened for the removal of big particles and stored at 4 °C. The sulphate (SO_4^{2-}) concentration was measured using a COD and Multiparameter Bench Photometer HI 83099 (Hanna Instruments Inc., USA). Electrical conductivity EC (mS/cm), pH and redox potential Eh (mV) were measured using a Lovibond SensoDirect 150 multi-parameter water quality meter. The pH was calibrated using reference buffer solutions before analysis. The methods used are analogous to the previous report except that samples were treated with 1% HNO_3 before metal ion analysis [26]. The metal ions concentration in the AMD samples were measured using the inductively coupled plasma optical emission spectrometer (ICP-OES) (ICP Expert II, Agilent Technologies 720 ICP-OES). The physicochemical characteristics of the AMD sample being treated are presented in Table 1.

2.2. Isolation and growth medium

A 0.5 L sterilised reactor containing a sterile modified Postgate medium B (80% v/v) [27] – see Table 2, was inoculated with 0.1 L of AMD subsequent to anaerobic incubation at 35 °C for 7 d. The medium containing microbial community changed to black-grey which indicated proliferation of sulphate reducing microbes [28]. Subsequently, 0.4 L sterile modified Postgate medium B was incubated anaerobically with 0.1 L of inoculum for 7 d in a new sterile reactor. The process was repeated thrice. All components were analytical grade.

Table 1. AMD sample quality parameters.

Parameter	Value
Temperature	20 ± 2 °C
pH	2.9 ± 0.2
Electrical conductivity	7.5 ± 0.5 mS/cm
Redox potential	229.5 ± 3.6 mV
Turbidity	145.0 ± 2.2 NTU
COD	426 ± 6 mg/L
Sulphate	8080 ± 10 mg/L

Table 2. Components of modified Postgate medium B.

Reagents	Amount
KH_2PO_4	0.5 g/L
NH_4Cl	1.0 g/L
Na_2SO_4	1.0 g/L
$CaCl_2$. $2H_2O$	0.1 g/L
$MgSO_4$	2.0 g/L
Yeast extract	1.0 g/L
Ascorbic acid	0.1 g/L
Thioglycolic acid	0.1 g/L
$FeSO_4$. $7H_2O$	0.5 g/L
NaCl	26 g/L
Sodium lactate	5 mL
pH	7–7.5

2.3. Experimental set-up and procedures

The experiments were carried out anaerobically in a glass reactor fitted with an overhead stirrer for constant mixing at 250 rpm. The reactors operated at 35 °C and a pH of 7 was initiated with 10 % inoculum in 0.8 L Postgate medium B for 21 d in a 1 L working volume. A fresh Postgate medium B was used to replenish a volume (70%) of broth drained from the bioreactors weekly. Subsequent to the proliferation of a viable culture, the operational mode was changed to continuous operation, with fresh 100 mL AMD being fed daily for 7 d. Thereafter, operated bioreactors were changed to operate in a batch mode for 14 d. Overall, a daily sampling regime was used to measure constituents and parameters deemed necessary for the experiments. The microbial growth at a wavelength of 600 nm was monitored in a GENESYS™ 10S UV-Vis spectrophotometer (Thermo Fisher Scientific™, Waltham, MA, USA). The control experiment was without the microbial consortium. All measurements were in triplicate and mean values reported.

The removal efficiency of the pollutants was calculated using the Eq. (4):

$$Removal\ (\%) = \frac{(C_o - C_f)}{C_o} \times 100 \quad (4)$$

Where C_o *and* C_f are concentrations (mg/L) in the fresh AMD and treated AMD, respectively.

2.4. Microbial community analysis

Samples of AMD before treatment (S_a) and after treatment (S_b) were analysed for microbial diversity. The genomic DNA of the bacterial community sample was extracted using a PowerBiofilm DNA kit (MOBIO Laboratories Inc., USA), according to the manufacturer's guidelines. The Polymerase Chain Reaction (PCR) amplification and sequencing were done using 341F (5' – CCTACGGGNGGCWGCAG – 3′) and 785R (5' – GACTACHVGGGTATCTAATCC – 3′) targeting V3 – V4 of the 16S rRNA genes. Sequencing was completed at a commercial NGS service provider,

i.e. Inqaba Biotechnical Industries (South Africa). The BLAST-based data analysis was performed using an Inqaba in-house developed data analysis pipeline. The raw metagenomic sequence was deposited with the National Centre for Biotechnology Information-=https://www.ncbi.nlm.nih.gov/sra/?term=SRP149893. The raw sequences were cleansed and operational taxonomic information were estimated using Ribosomal Database Project's (RDP) 16S database v16 (http://rdp.cme.msu.edu/index.jsp) which were further analysed for chimera check in USEARCH version 6.0 [29]. For each sample, Shannon index and Chao index at 3% cut off level were estimated in RDP database.

2.5. Biochemical analysis

Subsequent to the metagenomics analysis of treated AMD (S_b), S_b was subjected to a series of biochemical reactions in VITEK® 2 Compact 30 system (BioMérieux, France). Cycloheximide supplemented nutrient agar was used for culturable bacteria species from the remediated AMD in plates incubated at 37 °C for 24 h subsequent to sub-culturing for strain purification. Gram reaction, malachite green, and methylene blue staining techniques were used as preliminary identification methods of gram-negative and gram-positive bacteria, including bacterial with endospore, respectively. Further details of the biochemical reactions are as described in [30].

3. Results and discussion

3.1. Microbial community diversity and biochemical reactions

The microbial community distribution is shown in Figure 1. At the phylum level, *Firmicutes* was predominant (39%), followed by *Proteobacteria* (15.5%) while *Bacilli* (40.80%) were the most dominant microbial communities at the class level, in raw AMD sample (S_a). Similarly, *Firmicutes* (38%) and *Proteobacteria* (28.6%) were the most dominant at phylum level while *Bacilli* (35.7%) and *Actinobacteria* (16.7%) were prevalent at class level in the treated AMD sample (S_b). These findings corroborate the prevalence of *Firmicutes* in the microbial population of the AMD [20, 31, 32]. This implies that the *Firmicutes* especially *Bacilli* are adaptable enough to the extremely acidic pH of AMD, making them suitable biological agent in bioremediation of contaminated environment.

Table 3 shows the microbial diversity and richness indices. The values of Shannon and Chao indices for S_a were higher than that of S_b, an indication that the microbial species richness reduced during the remediation process in the reactor. This could be attributed to the anaerobic operating conditions which are not suitable for some microbial community. For both samples, the coverage was above 99% which suggest that the abundance analysis is a good representative of the microbial diversity.

Biochemical tests in combination with metagenomic analyses attested the dominance of *Bacilli* in the microbial population of the bioreactors used for AMD remediation. Albeit, identified species in VITEK® 2 compact system are predominantly facultative organisms such as *Bacillus amyloliquefaciens, B. atrophaeus, B. cereus, B. mycoides,* and *B. thuringiensis, B. smithii,* as well as *B. subtilis* which propagates anaerobically using nitrate as electron acceptor [33, 34]. The dominant *Bacillus* species were deposited with NCBI database. Detailed biochemical identification and their respective confidence level are shown in Table 4.

Furthermore, the microbial community indicated various substrates being utilised as expected. Prominent in the substrates were glycogen, D-galactose, pyruvate, Inulin, D-glucose, urea, saccharose/sucrose, acetate,

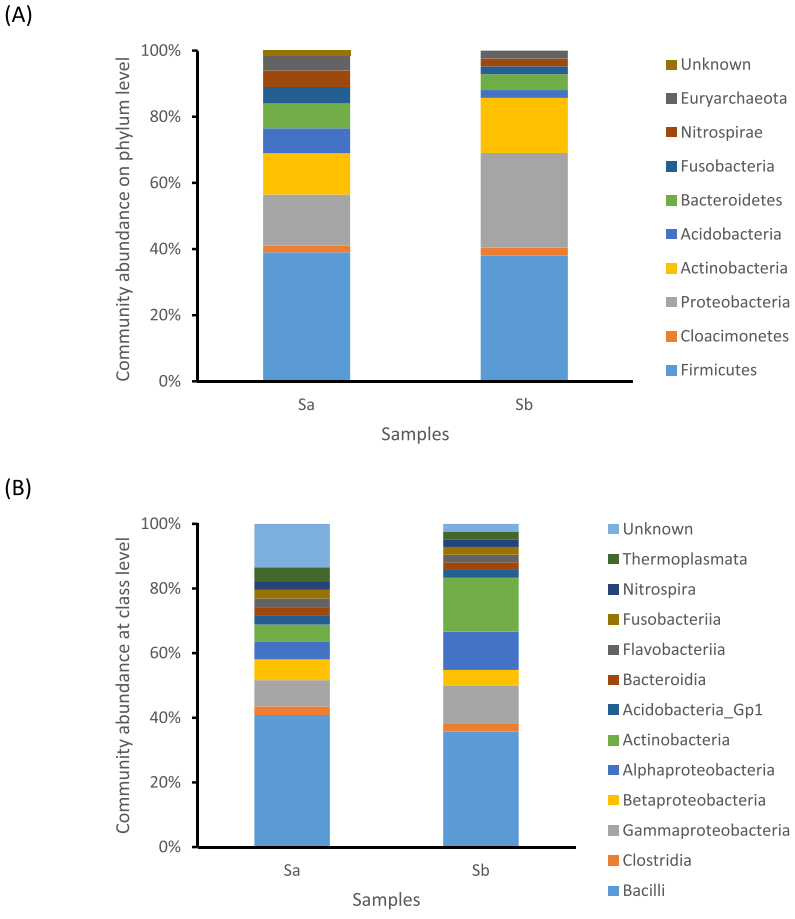

Figure 1. Community barplot analysis of raw AMD and treated AMD at phylum (A) and class (B).

Table 3. Microbial community diversity and richness indices of samples.

Sample ID	Cluster	Chao	Shannon	Coverage
S_a	123	3813	4.809	0.999
S_b	42	903	3.737	1.0

citrate, and DL-lactate, amongst others. Table S1 indicated that, contrary to the previous reports that limit the range of substrates as energy sources for organisms remediating acid mine drainage, the number of electron donors and acceptors was determined to vary [35, 36]. This supports the affirmation that more than a hundred compounds could serve as a potential substrate for microorganisms degrading AMD, due to the varying metabolic pathway under both aerobic and anaerobic conditions [37, 38, 39].

In addition, the dominance of aminopeptidase in the biochemical reactions was an indication of the consortium's ability to survive under nitrogen-limited conditions. Prominent among the enzymes are β-Xylosidase, Leucine-arylamidase, β-galactosidase, alanine arylamidase, tyrosine arylamidase, and α-Glucosidase, including phosphatase, which acts as biocatalyst for sulphate reduction [40]. In search of survival in a polluted environment, several heavy metal tolerant organisms have been shown to produce these metallo-aminopeptidases enzymes [41, 42]. The microbial community used in this study also showed resistance to several known inhibitors (Bacitracin, Kanamycin, Novobiocin, Oleandomycin, Optochin and Polymixin B) [43, 44].

3.2. Performance of the continuous reactor systems for AMD remediation

Several sulfidogenic reactors have been operated in diverse modes such as batch, continuous or semi-continuous with varying levels of performance. Singh et al. [45] reported 82% sulphate reduction in a static batch anaerobic reactor while 80 % removal efficiency was observed in a batch biofilm reactor [46]. Meanwhile, a continuous mode reactor with two-stage operations was the most widely reported to have a high remediation potential [5, 23, 47, 48]. In an up-flow anaerobic granular sludge bed (UASB), a 98% sulphate reduction was reported by Najib et al. [5] similar to the report of Dev et al. [47] in an up-flow anaerobic packed bed reactor. The reactor in this study was operated for 42 d at temperature 35 ± 2 °C and start-up pH of 7–7.5. Fresh AMD (8080 mg SO_4^{2-}/L) was introduced to the reactor on the 22nd day. The sulphate profile showed a steady rise in sulphate reduction until the end of continuous operation, together with microbial propagation – Figure 2. This reveals the effectiveness of the microbial community, taking into account the original concentration of sulphate in the raw AMD. Previous studies have always focused on synthetic wastewater with a lower sulphate concentration of less than 3000 mg SO_4^{2-}/L [13, 47, 49]. The relatively large residual sulphate concentration (1195 mg SO_4^{2-}/L) can be attributed to the slow rate of reduction in addition to high heavy metals concentration in the raw AMD which impeded the microbial activities. Due to the toxicity of heavy metal at higher concentrations, previous reports have shown that they inhibit microbial activities during sulphate reduction and thus

Table 4. Biochemical identification of microbial species in the AMD system.

Organism	Confidence level	Probability	Accession Number
Bacillus smithii	Excellent	98%	MT994646
Bacillus cereus	Excellent	98%	MT994644
Bacillus mycoides	Excellent	98%	MT994645
Bacillus thuringiensis	Excellent	98%	MT994648
Bacillus amyloliquefaciens	Good	90%	MN538986
Bacillus atrophaeus	Good	90%	MT994643
Bacillus subtilis	Good	90%	MT994647

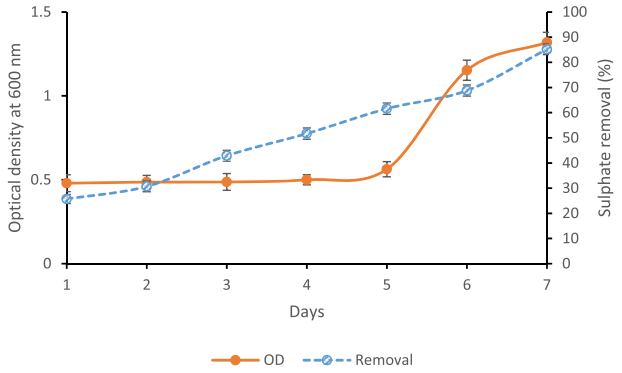

Figure 2. Microbial growth and percentage sulphate removal in continuous mode.

reduce their metabolism [23]. Furthermore, a high concentration of copper caused almost a 50% reduction in microbial removal efficiency [50]. After 7 d of continuous stirring and data capturing, the reactor was left in a static batch mode for 14 d, and sulphate concentration was found to have reduced to 60 mg SO_4^{2-}/L, representing a 99% removal efficiency which compared well with previous reports on AMD remediation. This study implied that the physicochemical properties of the microbial environment play a major role in heavy metal inhibition in sulphate reduction operating systems and that a single-mode operation is insufficient for the reduction of the sulphate in heavy metal-laden AMD.

A decline in pH was noticed at the beginning of the continuous operation mode most likely because of highly acidic raw AMD introduced – Figure 3. Most known sulphate-reducing bacteria grow at optimum pH between 6 and 8 [51]. At low pH, more energy investment is required for the migration of protons across cell membranes and less energy will be available for microbial growth. However, thermodynamic studies have shown that Gibbs's free energy of sulphate reduction is higher at lower pH resulting in more energy gains [52]. When this energy gains support proton migration, suitable and sustainable growth is achievable. The BLAST result of the sample sequences showed similarity with *Acidithiobacillus ferrooxidans* (0.09%) and *Acidiphilium* sp. (0.05%) which might have accounted for the little growth observed during days 1–2 when bioreactors operated in a continuous mode. Furthermore, there was an increase in redox potential (Eh) due to the higher Eh of the raw AMD – Figure 3. A steady decrease in the Eh and increase in pH was observed after 2nd day of continuous operation, suggesting an adjustment of the microbial community to the new

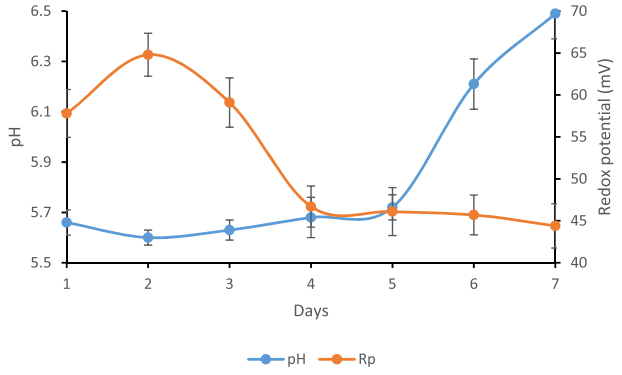

Figure 3. pH and redox potential profile of the microbial community during sulphate reduction.

Table 5. Effect of the microbial community on the heavy metals removal in the AMD.

Heavy metals	Raw AMD (mg/L)	After treatment (mg/L)	Ave. % Removal
Al^{3+}	484.7 ± 3.25	14.4 ± 0.85	97,03
As^{3+}	0.32 ± 0.02	0.11 ± 0.01	65,63
Cd^{2+}	**0.5 ± 0.11**	**0.009 ± 0.003**	**98,20**
Cu^{2+}	**0.46 ± 0.05**	**0.04 ± 0.001**	**91,30**
Cr^{3+}	0.13 ± 0.01	0.04 ± 0.001	69,23
Fe^{2+}	2308 ± 5.51	260.3 ± 2.78	88,72
Mg^{2+}	297.6 ± 2.67	132.5 ± 2.11	55,48
Mn^{2+}	**60.8 ± 1.89**	**3.15 ± 0.21**	**94,82**
Ni^{2+}	8.09 ± 0.56	1.75 ± 0.08	78,37
Pb^{2+}	**5.47 ± 0.43**	**0.32 ± 0.01**	**94,15**
Sr^{2+}	**1.0 ± 0.11**	**0.058 ± 0.002**	**94,20**
Zn^{2+}	7.93 ± 0.34	3.3 ± 0.13	58,39

Bold indicates microbial community almost removed heavy metal completely.

conditions. An identical drop in Eh and a rise in pH have been reported for the treatment of AMD [5, 23, 47].

Table 5 shows the initial and residual concentrations of heavy metal in the raw AMD prior and post-treatment with the microbial community, respectively. Heavy metals were removed in the form of metal sulphide precipitates. The microbial community reduces sulphate to sulphide which reacts with the heavy metal ions, resulting in insoluble metal sulphide precipitate. The highest metal removal efficiency was found in Cd^{2+} (98%) followed by Al^{3+} (97%). Removal percentages of 69, 66, 58 and 55% were observed for Cr^{3+}, As^{3+}, Zn^{2+}, and Mg^{2+}, respectively, with all other metals being removed above 70%. This performance could be attributed to the metallo-aminopeptidases activities in the presence of divalent metallic cations in the bioreactor. The existence of Mn^{2+}, Cu^{2+}, Zn^{2+} and Fe^{2+} has shown to alter the activities of metallo-aminopeptidases [40, 53, 54]. Previous reports have also shown that Cu^{2+}, Al^{3+}, Ni^{2+}, Pb^{2+} and Fe^{2+} are precipitated at acidic pH but could be precipitated at pH above 9.5 [55,56]. The influence of heavy metal tolerant facultative *B. cereus* in the microbial consortium as well as pH < 7 in the reactor facilitated the high metal removal in the AMD remediation. These results were similar to a report of a series of batch reactors and a floating column, whereby greater than 97% removal efficiency of heavy metals (Cd^{2+}, Zn^{2+}, and Cu^{2+}) were reported by the synergy observed between AMD degrading microbes and *B. cereus* [57], including a 99% removal of Al^{3+} in AMD using microbial consortium [58]. Although some reports have shown total abatement of heavy metals with sulphate reduction in the range of 80–90% [14, 59], the relatively high metal removal with sulphate reduction (85%) in this study can be enhanced by optimising process parameters to provide a kinetically suitable environment for the proliferation of the microbial community.

4. Conclusion

The results showed high sulphate reduction with heavy metal precipitation by a consortium of the microbial community in a continuously stirred tank reactor. After an adaptation period, sulphate reduction commenced and redox potential declined. There was an increase in pH while the dissolved concentrations of heavy metals were substantially reduced by the microbial community. The microbial group indicated the presence of both facultative and strictly anaerobic phylum *Firmicutes* was most useful in heavy metal precipitation and sulphate reduction in the reactor. The enrichment period (21 d) as well as the ability of the microbial community to consume various substrates as energy sources with the release of numerous aminopeptidases that catalyse AMD treatment enhanced the sulphate reduction and metal precipitation. This implies that several compounds could serve as a potential substrate for microorganisms degrading AMD, due to the varying metabolic pathway. These results indicate that this approach can be helpful in the design of efficient in situ AMD treatment.

Declarations

Author contribution statement

Enoch A. Akinpelu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Seteno K.O. Ntwampe: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Elvis Fosso-Kankeu & Felix Nchu: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Justine O. Angadam: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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