CHAPTER FIVE

CULTURABLE MICROORGANISMS ASSOCIATED WITH SISHEN IRON ORE AND THEIR POTENTIAL ROLES IN BIOBENEFICIATION

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Abstract

With one of the largest iron ore deposits in the world, South Africa is recognised to be among the top ten biggest exporters of iron ore mineral. Increasing demand and consumption of this mineral triggered search for processing technologies that can be utilised to “purify” the low-grade iron ore that contain high levels of unwanted potassium (K) and phosphorus (P). The need for a low cost and environmental friendly technology has therefore made biobenefication technology a potential process to solve this problem. This study investigated a potential biological method that can be further developed for a full biobeneficiation of low-grade iron ore minerals. Twenty-three bacterial strains that belong to *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobateria* were isolated from the iron ore minerals and identified with sequence homology and phylogenetic methods. Abilities of these isolates to lower the pH of the growth medium, high slime production and solubilisation of tricalcium phosphate were used to screen them as potential mineral solubilisers. Eight isolates were successfully screened with this method and utilised in shake flask experiments using iron ore minerals as sources of K and P. The shake flask experiments revealed that all the eight isolates have potentials to produce organic acids that aided the solubilisation of the iron ore minerals. In addition, all eight isolates produced a high quantity of gluconic acid but lower quantities of acetic, citric and propanoic acid. Scanning electron microscopy (SEM) and Fourier transform infrared (FITR) analyses also helped to uncover the role that biofilm and extracellular polymeric substances could play in mineral solubilisation.

5.1 Introduction

Iron ore is one of the most common minerals on the surface of the earth. The importance of iron is strongly linked to its hardness, durability, strength and ability to form alloys with other metals. These properties have made iron ore special and suitable for different applications in various industrial processes (Gutzmer *et al*., 2001; Beukes *et al*., 2003). Over the past few decades, the surge in the global demand for iron ore has led to increase production and exportation of this mineral by the iron ore-producing countries (Williams and Cloete, 2008; Delvasto *et al*., 2009). With this development, it is becoming increasingly difficult to find this mineral in its pure form. Iron ore mining companies are now faced with the challenges of refining and reprocessing low-grade iron ore minerals. Such poor quality iron ore minerals contain contaminants such as potassium, phosphorus, aluminium and sodium which are deleterious to the processing and products of this mineral (Parks *et al*., 1990; Yusfin *et al*., 1999; Williams and Cloete, 2008; Delvasto *et al*., 2009).

The type of unwanted elements in iron ore samples differs from country to country. For example, iron ore minerals of high P have been reported in Brazil, while Sishen mine in South Africa is having problems of high K (>0.24%) and P (>0.03%) contents of the iron ore minerals. Studies have revealed that these deleterious elements are always embedded in various associative minerals contained in the iron ore materials such as apatite, hematite, muscovite, quartz and goyasite (Parks *et al*., 1990; Williams, 2008; Delvasto, 2008). Both chemical and pyrometallurgical methods developed to solve these problems have not been generally accepted, because of cost and environmental concerns (Brombacher *et al*., 1997; Rawlings and Johnson, 2007). In addition, the development of an acceptable biological method of leaching iron ore has been slow due of the difficulty involved in extrapolating the available biomining procedures into this area of biohydrometallurgy. This is because most of the successfully concluded studies in biohydrometallurgy were based on the use of chemolithoautotrophic bacteria designed for bioleaching of sulfidic minerals. These are bacteria that are able to utilise both sulfur and iron cycles for the generation of their energy during bioleaching processes (Jain and Sharma, 2004;

Rawlings, 2005). Unfortunately, iron ore minerals such as Sishe iron minerals do not belong to this group (Jain and Sharma, 2004; Williams, 2008).

In similarity to bioleaching of sulfidic minerals, the importance of the unwanted elements as nutrients for microorganisms has encouraged scientists to develop methods that enable microbes to utilise them as sources of energy and other metabolic processes. These processes involve the use of bacteria and fungi, usually soil-associated, and some indigenous microflora that are capable of dissolving complex mineral materials. Solubilisation of the minerals is achieved through the production of metabolites that contain organic acids as the active ingredients (Parks *et al*., 1990; Deo and Natarajan, 1998; Pradhan, 2008; Williams, 2008; Delvasto, 2009). Both bacteria and fungi that were previously investigated for biobeneficiation of non-sulfidic minerals have been identified as organic acids producing-microbes. The process occurs through direct oxidation pathway where gram-negative bacteria are mostly involved. In this situation, organic acids produced in the periplasm could easily diffuse into adjacent environment and subsequently dissolve insoluble forms of minerals such as calcium phosphate (Goldstein *et al*., 2003). The production of organic acids by such microbes therefore provides a platform for ion exchange in forms of proton donation and complexation (Gadd, 1999; Jain and Sharma, 2004). A recent example was the solubilisation effects of gluconic acid released by *Burkhoderia caribensis* FeGL03 on the mobilisation of P from iron as reported by Delvasto *et al*. (2009). Furthermore, Williams (2008) also utilised citric acid obtained from *Aspergillus niger* for the biobenefication of Sishen iron ore. In addition, factors such as molecular functionalities of extracellular polymeric substances (EPS) produced by microbes and attachment of the microbes to mineral surfaces have also been indicated as important in iron ore solubilisation (Natarajan and Deo, 2001; Delvasto *et al*., 2009). In their study, Delvasto *et al*. (2009) reported EPS production by *Burkholderia caribensis* FeGL03 as partly responsible for the solubilisation of P from iron ore materials. All these attributes are the theoretical background of the present investigations of bacteria associated with iron ore and their roles in biobenefication of this mineral.

The ability of the isolates to reduce the pH of the growth medium was taken as an indication of medium acidification (Welch and Ullman, 1996) while those that dissolve water-insoluble tricalcium phosphate were assume to have the capability to produce high gluconic acid (Delvasto

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et al., 2009). The aims of this study were therefore to i.) isolate and characterise culturable bacterial population inhabiting the iron ore surfaces, ii.) screen the isolates in order to identify potential organic acids-producing bacteria through the use of microbial features - characterisitics such as ability to lower the pH of the growth medium, high slime production and dissolution of insoluble phosphorus were utilised, and iii.) investigate the biobeneficiation (K and P reduction) potential of the organic acids-producing isolates.

5.2 Materials and methods

5.2.1 Origin and preparation of iron ore samples

Two types of iron ore samples were collected from Sishen mine located in the Nothern Cape Province of South Africa. These samples were originally characterised by the company as KGT (conglomerates) and SK (shale). The iron ore materials were milled and sieved into sizes that are between <0.21 mm to >0.1 mm. Pretreatments of iron ore samples are as stated in chapter two (section 2.2.20). ICP was used to check any possible change in the P and K contents of the iron materials after this treatment. Dried samples were used in the leaching experiment as sources of K and P.

5.2.2 Preparation of media

Three different media were used for the isolation of bacteria in this study. This included a phosphate solubilising medium (PSM) (Mehta and Nautiyal, 2001), Nutrient agar (NA) (Biolab) and Typtone soy agar (TSA) (Biolab). The PSM contained $(NH_4)_2SO_4$, 0.10g/L; $MgSO_4{\cdot}7H_2O$, 0.25 g/L; $MgCl_2{\cdot}6H_2O$, 5.00 g/L; KCl, 0.20 g/L; $Ca_3(PO_4)_2$, 2.5 g/L; 10 g/L of glucose and agar, 20 g/L.

5.2.3 Isolation of bacteria from iron ore samples

A 5000-g sample of the iron ore materials was added to 1 L of de-ionised water inside autoclaved 2-litre beakers under sterile conditions and this was replicated three times for each mineral type. The beakers were covered with three-layer sterile foil paper and shaken at 60 rpm at room temperature. After 24 h of shaking, 10 ml of the homogenised liquid part of the mixture was taken from each beaker and replicates pooled together for each mineral type. This was followed by 3 min vortexing and serial dilution (10^{-1}, 10^{-2} and 10^{-3}). Eighty-microliter volumes of the diluted samples was inoculated onto PSM, NA and TSA plates using spread plate technique. Inoculated plates were incubated at 37 °C for 48 h.

Morphologically distinct colonies were identified and obtained from the plates. The colonies were suspended in 1 ml autoclaved double-distilled water. The suspension was serially diluted and the 10^{-2} dilution was plated out onto NA (Biolab) by spread plating of individual colonies. Distinct colonies were obtained after incubation at 37ºC for 24 h. Pure colonies were streaked onto NA as representatives of the individual bacterial isolates.

5.2.4 Screening of phosphorus-solubilising, potassium-solubilising and low pH- isolates

All the isolated bacteria samples were inoculated onto nutrient broth (NB) and incubated at 37 °C. The pH of the growing culture was checked after 24 and 48 h for any change. In addition, 10-µl volume of each isolate was inoculated at the centre of the PSM, containing (g/L): $MgSO_4{\cdot}7H_2O$ - 0.25; $(NH_4)_2SO_4$ - 0.10, $MgCl_2{\cdot}6H_2O$ - 5.00; KCl - 0.20; $Ca_3(PO_4)_2$ - 2.5; glucose-10 and agar – 20, was used for the selection of phosphate-solubilising bacteria. For isolation of potassium-solubilising isolates, a medium containing (g/L): starch - 10, $Na_2HPO_4.12H_2O$ -2, $FeCl_3$ – 0.005, $MgSO_4.7H_2O$ - 0.5, $CaCO_3$ -0.1, Yeast extract – 1 and agar – 20 (at pH 7.4), was used (Lin *et al*., 2001).

5.2.5 Molecular identification of the isolates

Genomic DNA extraction was carried out using the Zymo Rresearch Fungal/Bacterial DNA Kit™ (Cat.# 6001) according to the manufacturer's instructions. The 16S rDNA bacterial genes was the target region for the PCR amplification using a universal pair of bacterial forward and reverse primers; GM5F (5´-CCTACGGGAGGCAGCAG-3´;Tm-58.2ºC) and R907 (5´CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCGTCAATTCCTTTGA GTTT-3´;Tm-1.8ºC) (Muyzer, *et al*., 1995) respectively. PCR was conducted in a 50µl reaction which contained the following: 0.4µM of each of the primers, 1.25 U of *Taq* polymerase, 5 µl of Promega 10X buffer (0.2 mM) promega dNTPs, 1.75 mM of Magnessium chloride and 2µl template DNA. The PCR was performed on a MJ Mini Personal Thermal Cycler (Bio-Rad) using these conditions: initial denaturing at 94 °C for 2 min, followed by 4 cycles of 30 s at 94 °C (denaturing), 45 s at 68 °C (annealing), 2 min at 72 °C (elongation). These steps were repeated

(excluding initial denaturing) with decreasing annealing temperature at 66, 64, 62, 60 and 58 °C running at 4 cycles, except at the annealing temperature of 58 °C that ran at 12 cycles. Final elongation was at 72 °C for 8 min. The different annealing temperatures listed in decreasing order were due to the high variation in the T_m of the two primers. PCR products were separated electrophoretically with ethidium bromide (0.1µg/ml)-stained 1% agarose gel running at 120V for 1 h. DNA was visualised and photographed using a Uviprochem Transilluminator.

Cleaning of the PCR products obtained was done using the PROMEGA Wizard SV Gel and PCR purification kit (Cat.# A9280) and resuspended in 30 µl of nuclease-free double distilled water. Cleaned PCR product was sent to the Inqaba Biotechnical Industries (Pty) Ltd Sequencing Facility. Forward and reverse sequences of the 16s rDNA regions obtained were aligned to obtain consensus sequence using BioEdit software prior to BLASTing. Homology sequences were thereafter compared on the NCBI website (Hall, 1999) to confirm the nearest identical organism.

5.2.6 Phylogenetic analysis

Nucleotide sequences of two bacterial isolates (closest to the isolated strains) for each isolate were obtained from the GenBank. Meanwhile, some of the isolates shared close relatives. All the bacterial sequences obtained were aligned using ClustalX software (Thompson *et al*., 1997). Further alignment was carried out with online version of MAFFT software (Katoh *et al*., 2002). The phylogenetic analyses were carried out using Mega 4 software and the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2007). Using *Salmonella paratyphi* as an outgroup, neighbour joining (NJ) method (Saitou and Nei, 1987) was performed to infer the evolutionary history of the isolates and the bootstrap consensus tree inferred from 1000 replicates. All positions containing gaps and missing data were eliminated from the dataset.

5.2.7 Leaching experiments

Bacteria with lowest pH values and those that produced visible halos on PSM were selected for the leaching experiment. Isolates were inoculated into Nutrient Broth (NB) (Biolab) and grown at

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37 °C overnight. The bacterial cultures were then centrifuged at 13000 rpm after which the supernatant was discarded. The cells were then resuspended in autoclaved deionised water. Concentrations of all the bacterial isolates were adjusted with sterile water using a Beckman spectrophotometer (Du® 530) at OD_{600} to 0.1. Biobeneficiation experiment was conducted in a 100-ml Erlenmeyer flasks containing 5 g of iron ore mineral and 50 ml of modified PSM that contained $(NH_4)_2SO_4$, 0.10 g/L; $MgSO_4 \cdot 7H_2O$, 0.125 g/L; $MgCl_2 \cdot 6H_2O$, 2.5 g/L; KCl, 0.10 g/L; 2.5 g/L; 10 g/L of glucose and agar, 20 g/L. One milliliter of the adjusted concentration of the bacterial culture was inoculated onto the contents of the flask and incubated at 37 ºC for 3 weeks. The experiment was in triplicate and harvesting was done at weekly intervals. There were two control treatments – the first control had the iron ore and the PSM but no bacteria, while the second had the PSM and the bacteria but no iron ore.

5.2.8 pH measurement and high performance liquid chromatography (HPLC)

During harvesting, iron ore samples was separated from the spent medium by decantation. Spent broth from bacterial cultures were homogenised by vortexing, then centrifuged for 180 s at 16060 rpm and the supernatant frozen at –40 ºC prior to analysis by High Performance Liquid Chromatography (HPLC). This was later passed through the filter paper (0.22 µm) to remove any remaining particles from the medium of growth. Prior to the storage, part of the supernatant were used for pH measurement. Organic acids were separated with an Agilent Zorbax SB-Aq (4.6 x 150nm) 5-µm column, eluted isocratically at 1 mL min^{-1} with 20 mM NaH_2PO_4 at pH2 buffer with the column at 25 °C and detected on a diode array detector at 210 nm (Agilent 1100 series). Peak identity and organic acid quantity were determined by comparison with standards. The organic acid standard included gluconic, acetic, citric acid and maleic acid that were well separated under the described chromatographic conditions.

5.2.9 Fourier transform infrared (FTIR) spectroscopy

Exopolymeric substances produced by some of the bacterial isolates were analysed using reflectance infrared (IR) method with a KBr matrix. Measurements were taken with scans using a Perkin Elmer spectrum RX IFT-IR system. The process involved the initial precipitation of the

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spent medium with ethanol. A control treatment with the same culture medium and iron ore but with no bacteria was treated the same way and used as a background to set the machine. This was to enable the elimination of any interference from the culture medium and iron ore samples during the reading. The acquisition of the spectra was through the transmission mode. Pellets formed after the ethanol treatments were mixed with KBR and dried for 24 h at room temperature. This was followed by crushing and pressurisation of the mixture to form pellets that were used for the reading. Background was set with the control.

5.2.10 Microscopy

Part of iron ore samples collected during harvesting were fixed by applying 15 ml of fixing solution (2.5% glutaraldehyde in 0.0075 M phosphate buffer) onto the ore inside Greiner tube. Ore samples were washed three times for 15 min each with 0.0375 M phosphate ($Na_3(PO_4)$) buffer. The samples were then dehydrated at different alcohol concentrations (50, 70, 95, 100%), at 10 min each. The dehydrated samples were repeatedly soaked in 100% alcohol twice. This stage was followed by drying of the samples that were later sputter-coated in a Polaron Equipment LimitedSEM Coating Unit E5200 with gold prior to observation under the scanning electron microscope (SEM). They were then assembled for observation under the microscope at 5 kV on a JEOL 5800LV scanning electron microscope (Tokyo, Japan).

5.2.11 Induction Coupled Plasma (ICP)

Iron ore samples collected during harvesting were repeatedly washed with 0.1 M HCl and later left in deionised water for 24 h. The samples were dried at 104 °C before sending them for Induction Coupled Plasma (ICP-OES Optima 4300 DV, Perkin Elmer, Waltham, MA, USA) analysis by UIS Analytical Services, Pretoria, South Africa.

5.2.12 Experimental design and Statistical analyses

The statistical analyses were carried out using SAS software, version 9.2 (SAS Institute, 2008, Cary, NC, USA). The analyses was done as 3-way Anova with two levels of iron type (KB, SB),

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nine levels for bacteria including the control with no bacterial sample (KU1, KU7, KC1, KC2, KU6, KU8, SU5, SU7 and CTR-control) and three levels for the time (week)(Week1-W1, Week 2-W2, Week 3 W3). For all the variables, there were two types of control that were included (one with the bacteria and no iron ore and the other with no bacterial sample). The analysis was also done as a 3-way Anova with 3 levels of iron (KB, SB, CT-control), bacteria (9 levels): (KU1, KU7, KC1, KC2, KU6, KU8, SU5 and SU7) and three levels for the time; week (W1, W2, W3). For all these variables, the log transformation was used in order to fulfil the assumptions of the model and each time, an interaction of order 3 was observed, i.e. an interaction between IRON vs Bacterial vs Week. Multiple comparisons were done using the stepdown Bonferroni method in order to protect the type 1 error rate. Normality assumptions were verified with the Shapiro-Wilk's statistic and the homogeneity of variances was verified by the residual plots.

5.3 Results

Phosphorus and potassium contents of the iron ore materials were analysed by Induction Coupled Plasma ICP-OES Optima 4300 DV (Perkin Elmer, Waltham, MA, USA), which showed that KGT originally contains an average of 0.805% K and 0.14% P whereas SK has an average of 0.423% K and 0.09% P. Other major compounds contained in the iron ore samples are SiO_2 (32.48%), Al_2O_3 (4.12%) and Fe_2O_3 (61.51%) for SK, while KGT had SiO_2 (5.32%), Al_2O_3 (2.94%) and Fe_2O_3 (89.75%).

From the three media used, the highest number of isolates was obtained from the MMN medium, followed by TSA and then PSM. A total of 23 morphologically distinct isolates were obtained during the isolation processs (Fig. 5.1). The homology sequence (Appendix III) and phylogenetical analyses of the 16S rDNA of these isolates enabled their division into four different clades which included *Protebacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* (Fig. 5.1). Most of the isolates belong to the *Protebacteria* clade which was subdivided into Alpha-*Protebacteria* with isolate TKU1, Beta- *Protebacteria* with isolates KC2, KU2, KU13, SC4, SU4 and SU9 and Gamma- *Proteobacteria* with isolates KU6 and SU7. The *Actinobacteria* cluster consists of isolates KU8, SU3, KU4, KU7, KU3, KC4 and SU1, while the *Firmicutes* cluster consists of SU2, KC1, KU5, SU5, KU1 and SC5. Only one isolate (TS4) belongs to the *Bacteroidetes* clade.The isolates, together with their recently allocated accession numbers, as well as their close relatives from the GenBank are presented in Fig. 5.1.

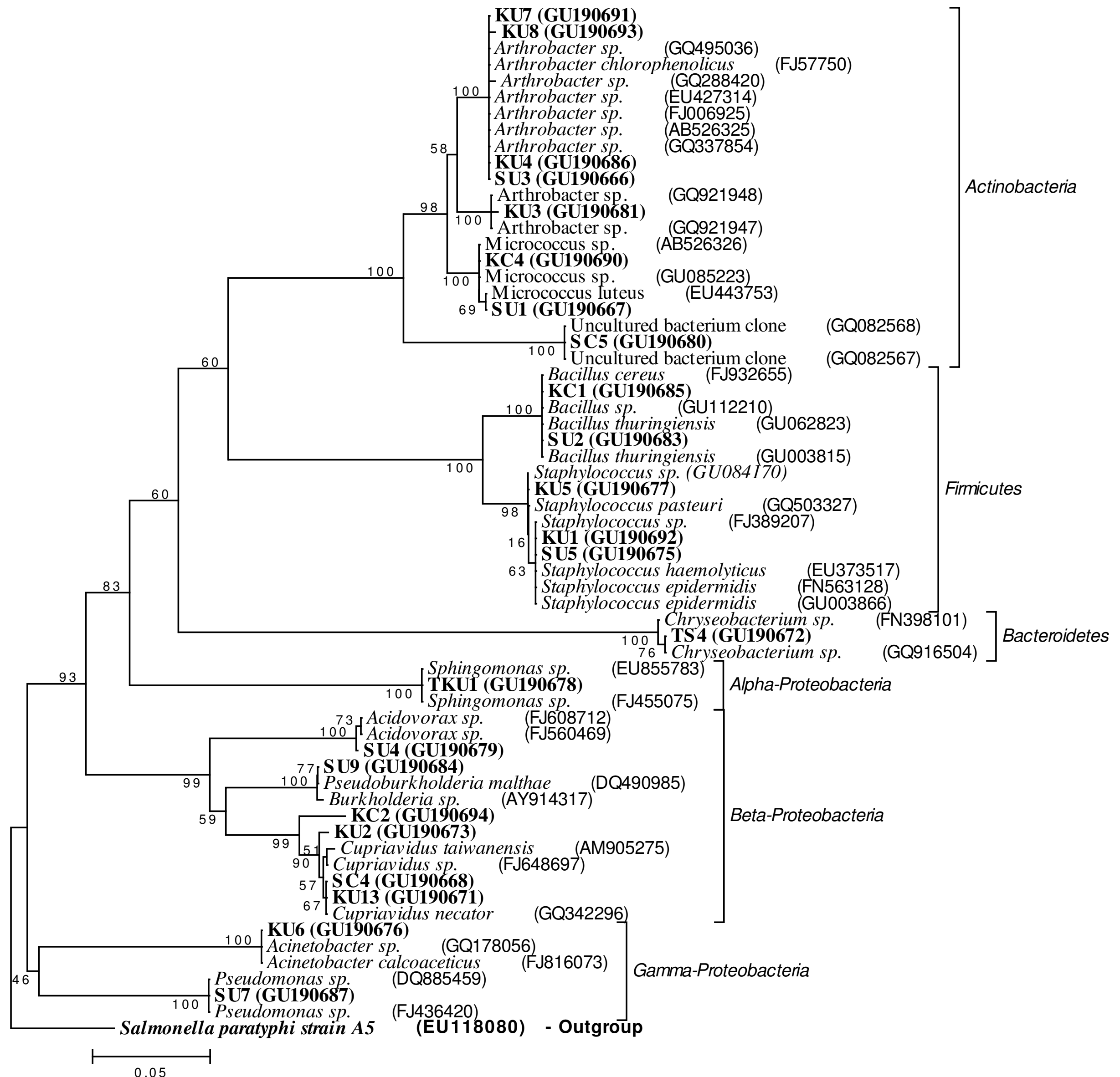

Figure 5.1: Phylogenetic tree of the 16S rDNA of bacterial isolates obtained from KGT and SK mineral types (in bold) and their related species obtained from the GeneBank as established by boostrap neighbor-joining method.

When cultured on PSM, seven of the bacterial isolates tested positive for the P solubilisation (Fig. 5.2). These include isolates KU6, SU5, SU7, KC2, KU8, KU7 and KU1. In addition, only one of the isolates effectively lowered the pH of the medium of growth towards the acidic range -

KC1. For the K solubilisation, four of P solubiliser isolates also showed positive abilities to solubise K by producing high levels of slime. At the end, eight bacterial isolates were positively identified as potential mineral solubilisers with these methods. Molecular and phylogenetic analyses of the nucleotide sequences of these isolates revealed that they are closely related to six genera that included *Staphylococcus* (KU1 and SU5), *Bacillus* (1), *Arthrobacter* (KU8 and KU7), *Acinetobacter* (KU6), *Cupriavidus* (KC2) and *Pseudomonas* (SU7) (Fig. 5.1).

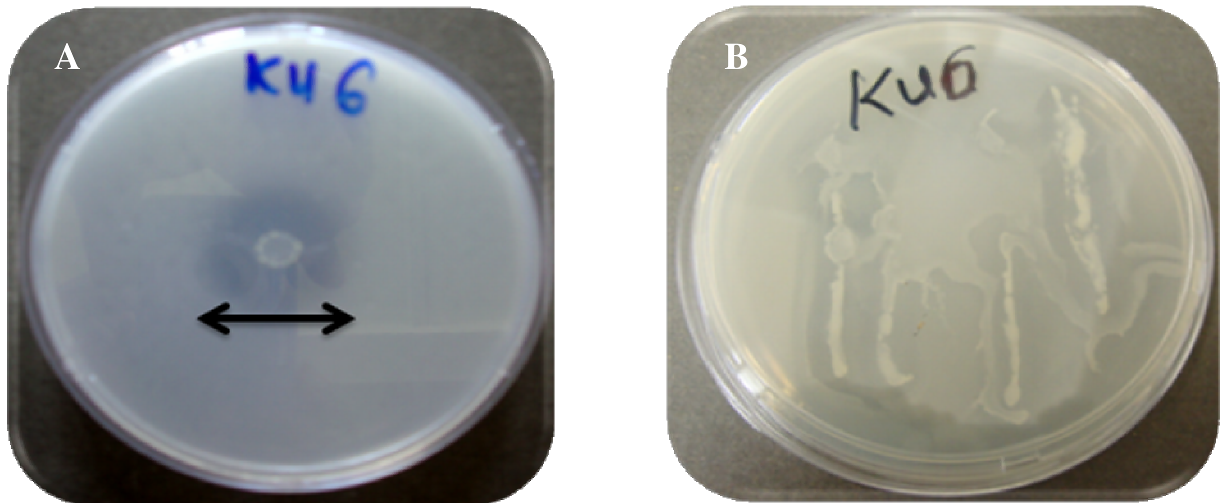

Figure 5.2: Phosphate-solubilising ability of isolate KU6 (A), as indicated by the halos (length of halo showed with the arrow) and high potassium usage, as indicated by high slime production that covered almost the entire plate.

The results of the shake-flask experiment revealed that iron ore type, bacteria type and time, as well as most of the interactions between these factors have significant effects on the rate of K and P removal from the iron ore (Table 5.1).

Table 5.1: Influence of mineral type, iron type, bacterial type and their interactions on the percentage K and P loss.

Sources of Variation	df	% K loss df= 105		%P loss df= 104	
		F	*P*	*F*	*P*
Iron	**1**	1650.2	<.0001	1684.52	<.0001
Bacteria	9	351.5	<.0001	1887.33	<.0001
Iron vs Bacteria	9	156.3	<.0001	555.4	<.0001
Week	2	34.29	<.0001	8.78	0.0003
Iron vs Week	2	3.08	0.0502	34.99	<.0001
Bacteria vs Week	18	14.65	<.0001	15.19	<.0001
Iron vs Bacteria vs Week	18	11.33	<.0001	21.48	<.0001

P-values < 0.005 are considered significant.

Higher percentages of K and P removal were obtained from SK mineral treatments compared to KGT mineral treatments (Fig. 5.4 and 5.6). Treatments involving isolate KU6 turned out to show highest percentage of K removal (up to 52%K removal) from SK mineral type (Fig. 5.4). Other isolates such as KU8, SU5, KU7, KC1 and SU7 also showed significantly higher K removal (>30%) (Fig. 5.4). For KGT mineral type, 29.52% was the highest percentage of K removed by bacterial isolate SU5 and there was no significant difference between this value and those from bacterial isolates KU6, KC2 and SU7 for this mineral type (Fig. 5.3). Potential for P removal (compared to K) seems to be more feasible for some of the isolates, as up to 97.5% of the total P was removed by isolate KU8 from SK mineral type (Fig. 5.6). More than 85% P was also removed from treatments involving isolates KU7, KU6 and KC2, with no significant difference among the values (Fig. 5.6). These isolates (KU8, KU7, KU6 and KC2) are also responsible for the highest P removal from KGT mineral type, removing more than 60% P from this mineral type (Fig. 5.5). In terms of time effect on K and P removal, there was no general trend that is applicable to all the isolates. For instance, isolate KU6 was able to remove a high portion of the K content of SK mineral type for the duration of the experiment (1st to 3rd week), whereas the value of percentage K removed by KU7 dropped from first week through second to third week (Fig. 5.4). Organic acid production by these isolates was significantly affected by mineral type, bacteria type and time, as well as the interactions between these factors (Table 5.2).

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Table 5.2: Three-way analysis of variance (ANOVA) with F and P values that that show the effects of bacterial type, mineral type and their interactions on the release of four different organic acids.

Sources of Variation	df	Gluconic acid df= 103		Acetic acid df= 102		Citric acid df= 106		Propanoic acid df= 107	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Iron type	2	7438	<.0001	3457.58	<.0001	192.63	<.0001	3153.09	<.0001
Bacterial type	7	2231.6	<.0001	865.68	<.0001	351.2	<.0001	389.48	<.0001
Iron type vs Bacterial type	15	466.49	<.0001	1066.8	<.0001	91.9	<.0001	295.52	<.0001
Time (Week)	2	199.6	<.0001	394.11	<.0001	86.03	<.0001	265.85	<.0001
Iron type vs Time (Week)	4	110.46	<.0001	78.83	<.0001	18.12	<.0001	149.08	<.0001
Iron type vs Bacterial type	16	3.01	4E-04	65.47	<.0001	8.83	<.0001	30.71	<.0001
Iron type vs Bacteria vs Week	32	6.12	<.0001	100.56	<.0001	9.61	<.0001	48.21	<.0001

P-values <0.005 are considered significant.

In general, gluconic acid was constantly detectable and the highest produced organic acid by the isolates. However, there are some discrepancies between the quantity of organic acids produced and leaching of K and P by the isolates. More gluconic acid production was recorded in some of the control treatments (Fig. 5.7). In terms of the two minerals, isolates growing under the treatments involving SK mineral type were able to release more acids, especially gluconic acid, than those growing under the KGT mineral treatments. Generally, there is increase in organic acid production by most of the isolates from 1st to 3rd week, but this does not portend the trend of K and P removal, as the rate of these elements removal in most cases decreases after the 2nd week of the experiment.

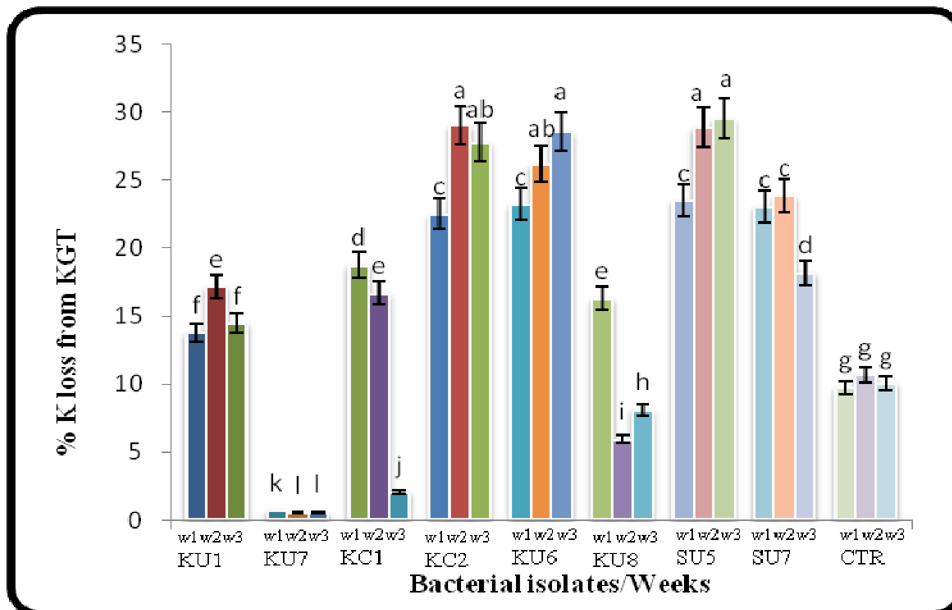

Figure 5.3: Amount of K loss from the KGT mineral type in treatment by all the identified isolates that are "mineral solublisers". Error bars are ±SE (n = 3). CTR represent where iron ore materials were left inside the modified PSM medium with no bacterial inoculation. (P<0.001).

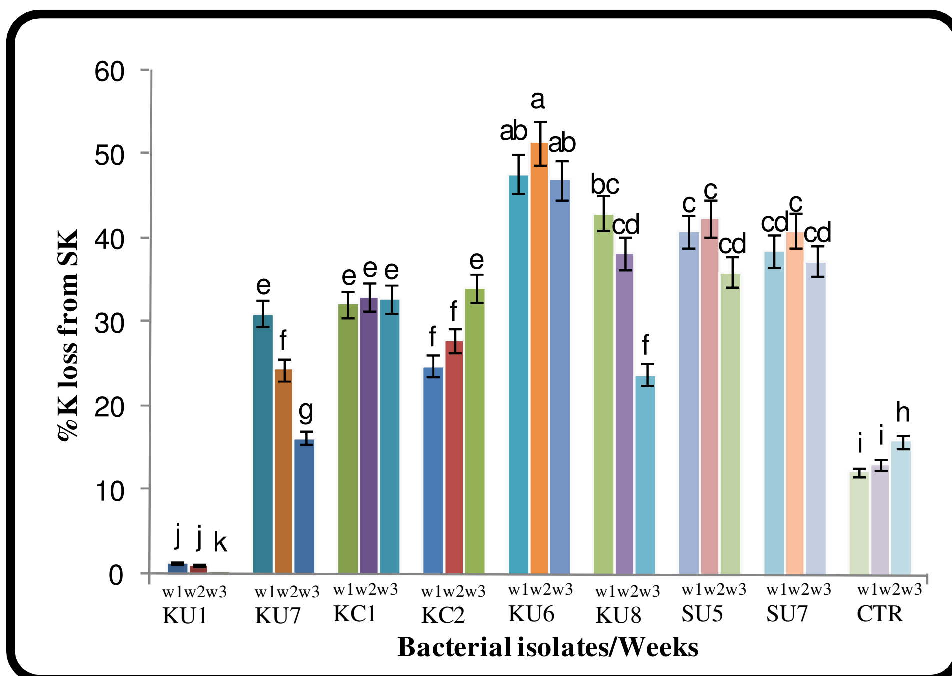

Figure 5.4: Amount of K loss from the SK mineral type in treatment by all the identified isolates that are "mineral solublisers". Error bars are ±SE (n = 3). CTR represent where iron ore materials were left inside the modified PSM medium with no bacterial inoculation. (P<0.001).

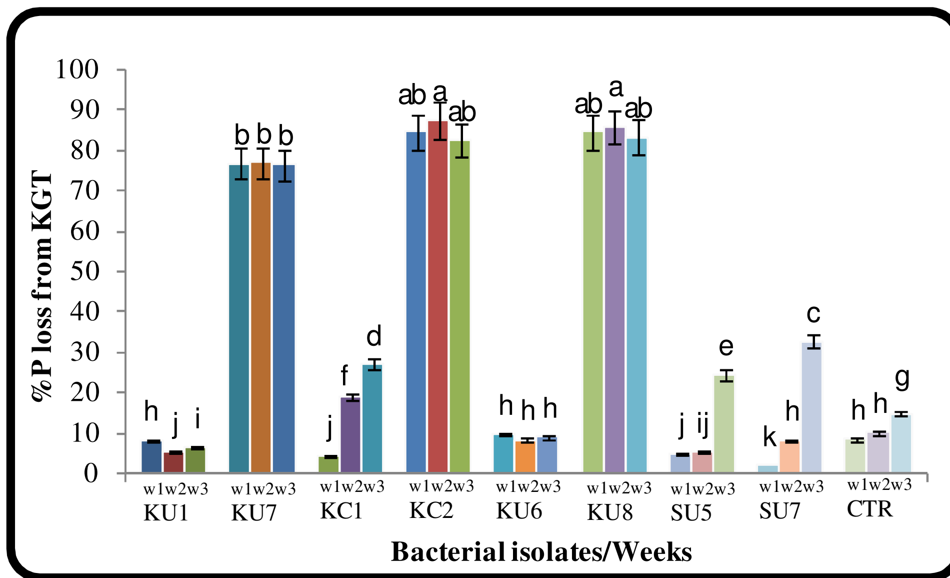

Figure 5.5: Amount of P loss from the KGT mineral type in treatment by all the identified isolates that are "mineral solubilisers". Error bars are ±SE (n = 3). CTR represent where iron ore materials were left inside the modified PSM medium with no bacterial inoculation. (P<0.001).

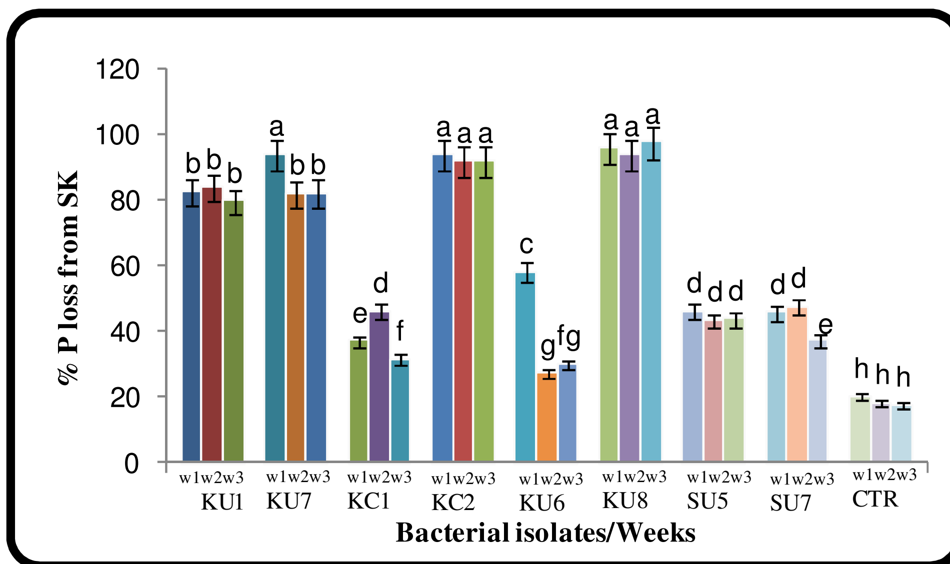

Figure 5.6: Amount of P loss from the SK mineral type in treatment by all the identified isolates that are "mineral solublisers". Error bars are ±SE (n = 3). CTR represent where iron ore materials were left inside the modified PSM medium with no bacterial inoculation. (P<0.001).

Statistical analysis of the pH result indicates a strong influence of the mineral type on the pH (Fig. 5.8). It is apparent that mobilisation of elements from SK mostly occurred at pH above 5 while for KGT it is mostly below pH 5. Few isolates that showed potential to reduce K and P from the ore minerals were selected for microscopic observations. Biofilms of isolates KU6, KU8, SU7, KU7 and SU5 were observable during the SEM analysis (Fig. 5.10A to 5.10E). The biofilm formation was at different intensity with more visible attachments observed for isolates KU6, SU5 and KC2. Isolate KU8 was observed to produce EPS, with very strong binding capacity that influenced the sticking together of a larger quantity of the mineral particles (Fig. 5.10). The FTIR spectra of precipitated substances showed several absorbance bands (Fig. 5.10) from isolates KU6 (best for K removal) and KU8 (best for P removal). As listed in Table 5.3, these bands depict different functional groups of proteins, lipids extracellular polysaccharides and nucleic acids.

Table 5.3: Band assignments of FITR spectra obtained from experiments involving isolates KU6 and KU8.

Functional groups (Reference)	Isolate KU6	Isolate KU8
OH of water (Omoike and Chorover, 2004; Delvasto *et al.*, 2009)	3410	
Asymmetric -CH3 stretching vibration, fatty acids (Omoike and Chorover, 2004; Delvasto *et al.*, 2009)	2968	2982 2958
Asymmetric -CH2 stretching vibration, fatty acids (Omoike and Chorover, 2004; Delvasto *et al.*, 2009)	2908 - 2364	2858
Carboxyl (C=O) stretch; Ester, fatty acids (Jiang *et al.*, 2004; Delvasto *et al.*, 2009)		1728
Amide I (C=O)	1657	
Amide II, N-H, C-N and structure of proteins (Jiang *et al.*, 2004; Delvasto *et al.*, 2009)	1537	1530
Carboxylate (Jiang *et al.*, 2004; Delvasto *et al.*, 2009)	1401	1402
C-O bond from carboxylate ions (Omoike and Chorover, 2004)	1314	
Vibrations of -COOH and C-O-C in ester (Jiang *et al.*, 2004; Delvasto *et al.*, 2009)	1234	
P=O bond stretching in phosphate (Jiang *et al.*, 2004; Delvasto *et al.*, 2009)	1218	1201
C-O-C (Glycosidic linkage), C-O, C-C vibrations (polysaccharides) (Omoike and Chorover, 2004; Jiang *et al.*, 2004; Delvasto *et al*, 2009)	1111 - 1025	1141-1070
Phosphate or sulfur functional groups (Comte *et al.*, 2006 Delvasto *et al.*, 2009)	≤ 903	≤ 974

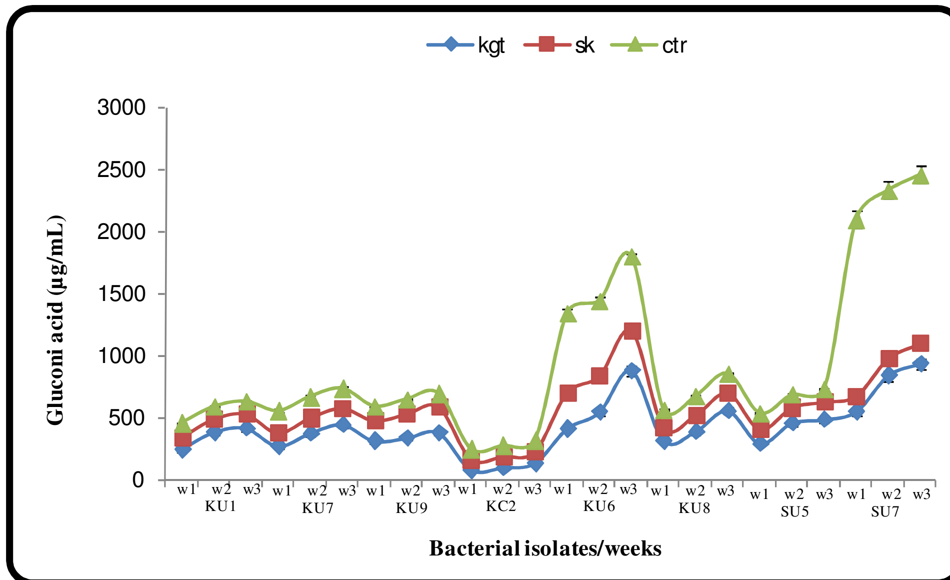

Figure 5.7: Amount of gluconic acid released during the shake flask experiment in the presence of both KGT and SK mineral types. CTR represent where bacterial isolates were cultured with PSM (complete), and no iron ore was present. (P<0.001).

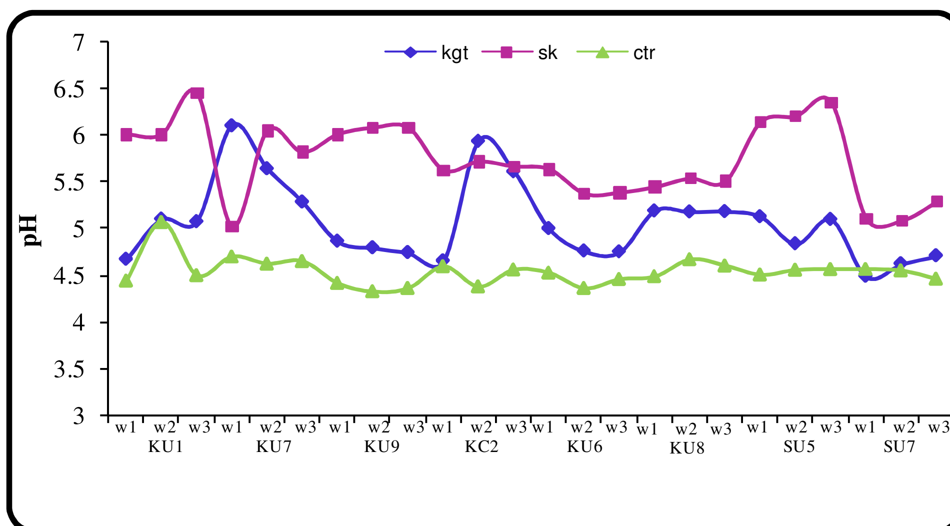

Figure 5.8: pH measurements during the shake flask experiment in the presence of both KGT and SK mineral types. CTR represent where bacterial isolates were cultured with PSM (complte), no iron ore was present. ($P<0.001$).

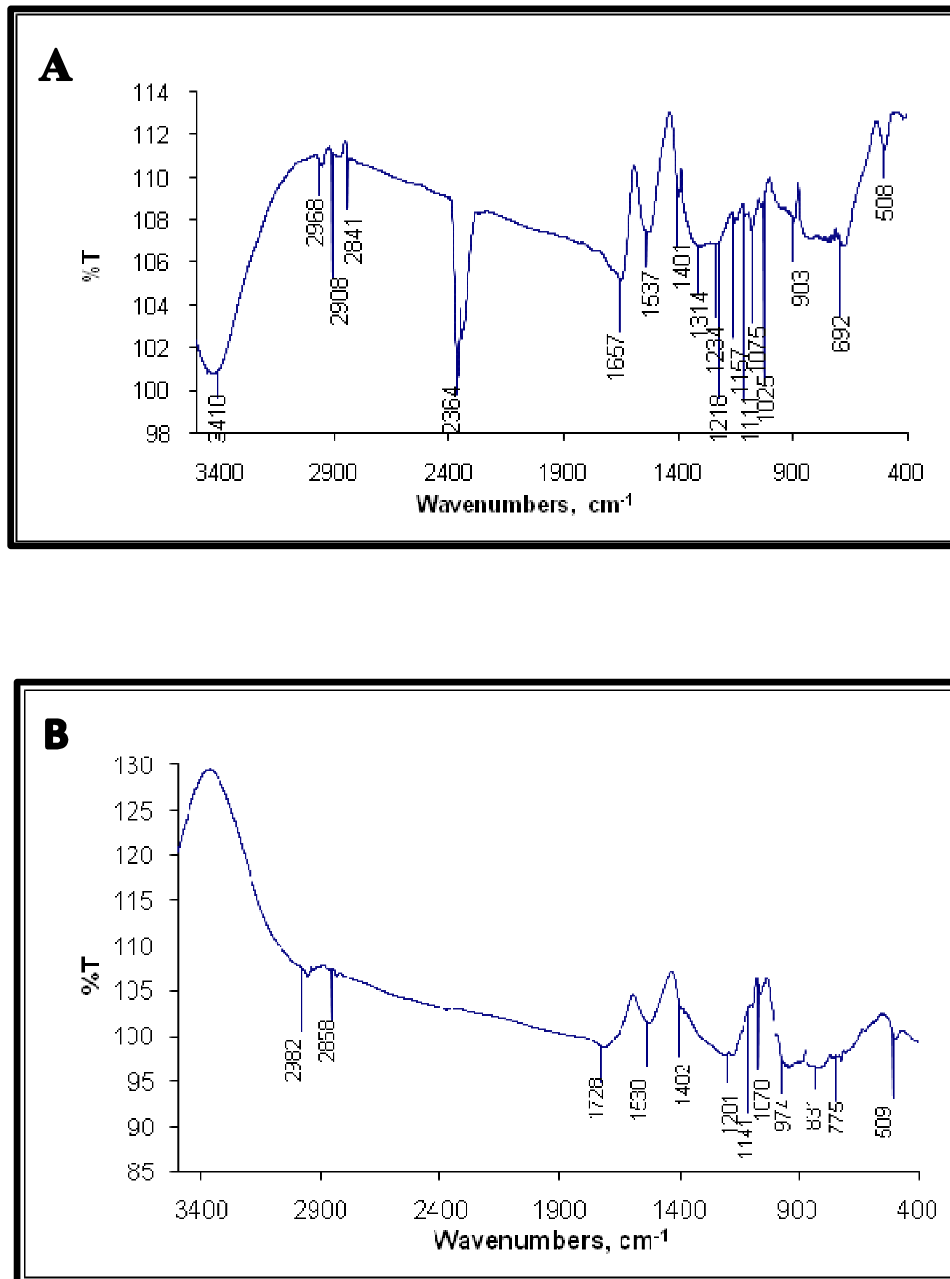

Figure 5.9: FT-IR spectrum of precipitated bacterial EPS from isolate KU6 (A) and isolate KU8 (B) growing in the presence of SK mineral type, week 1.

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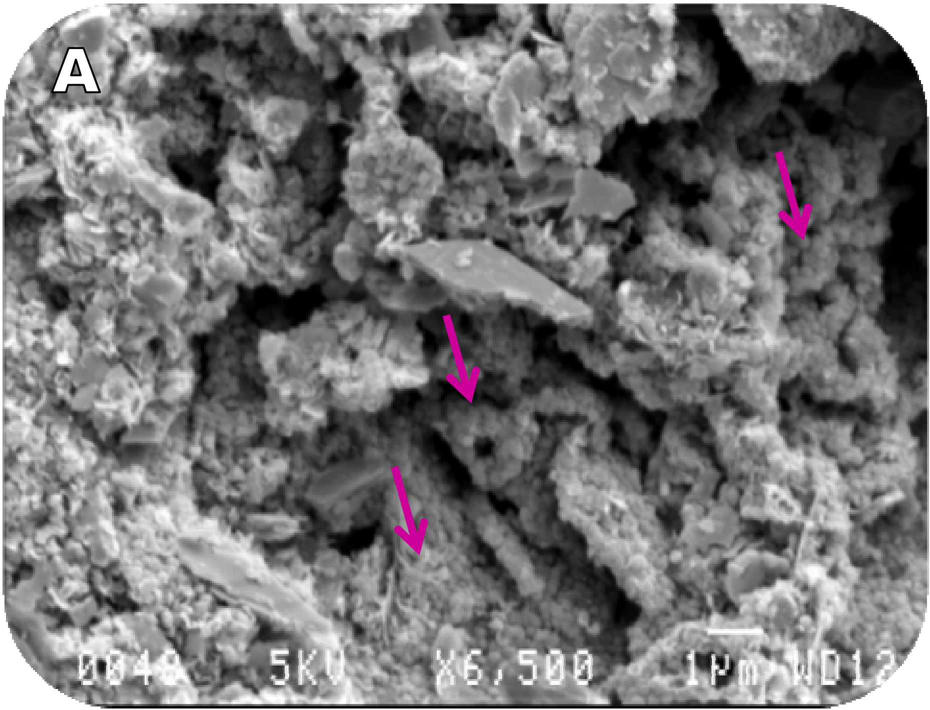

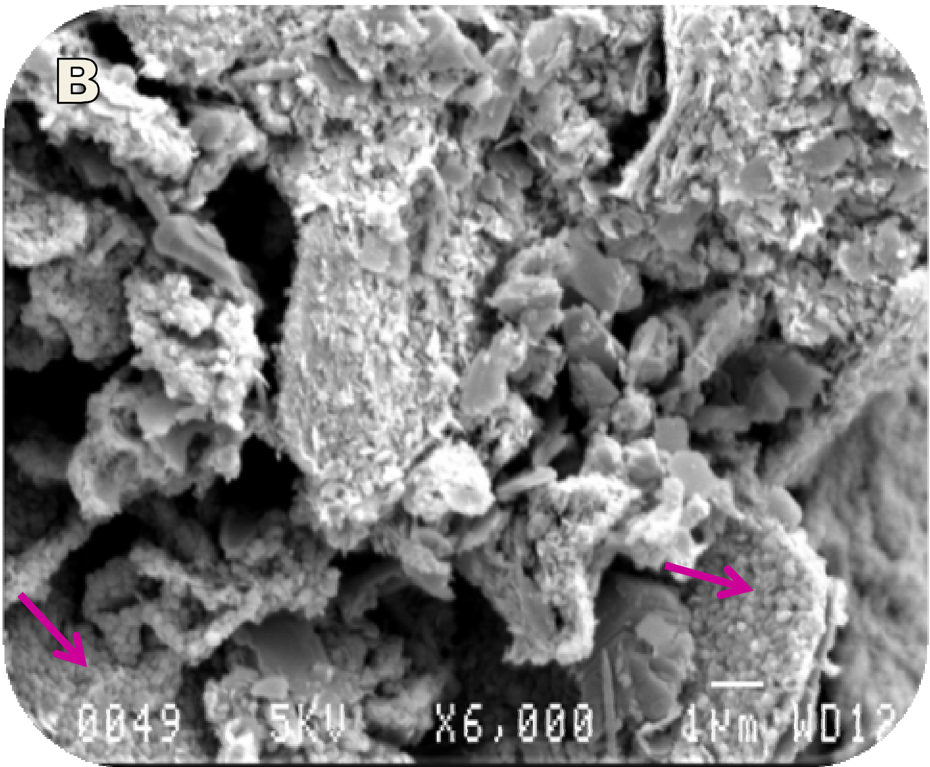

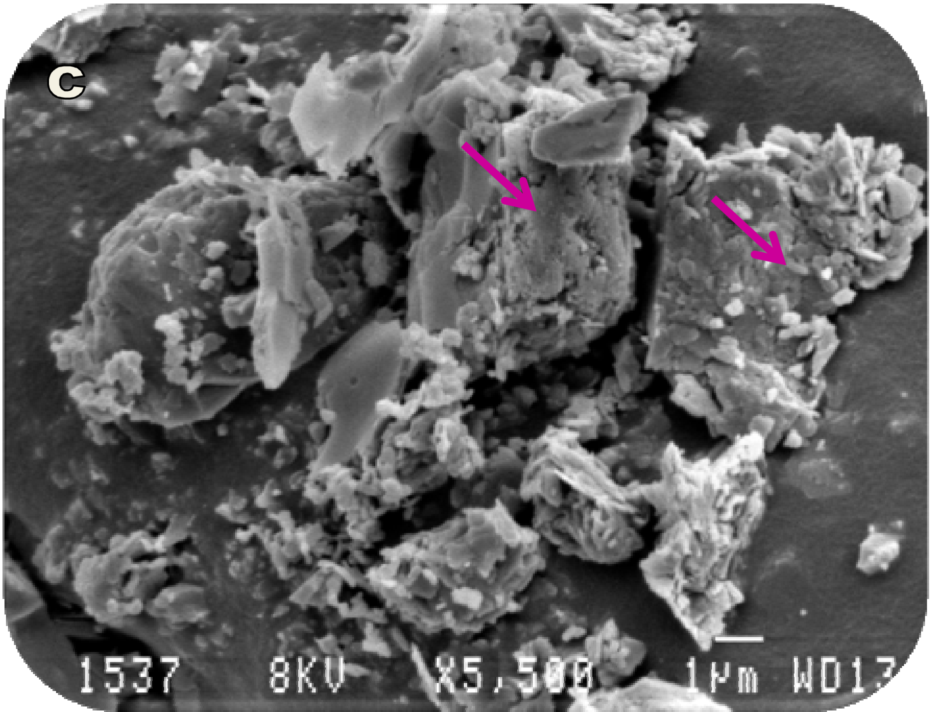
C
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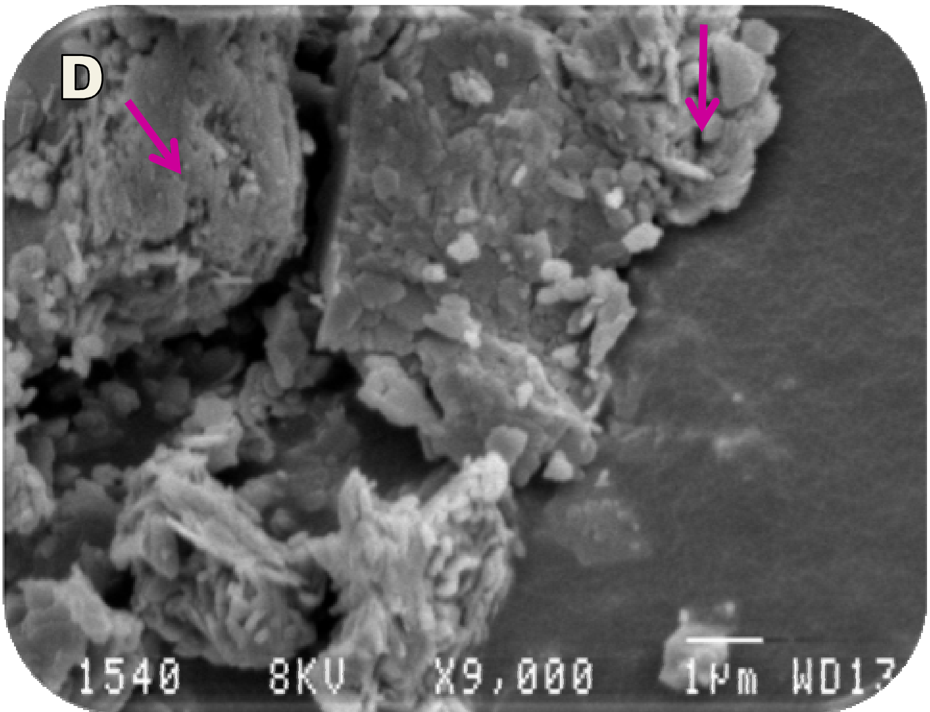
D
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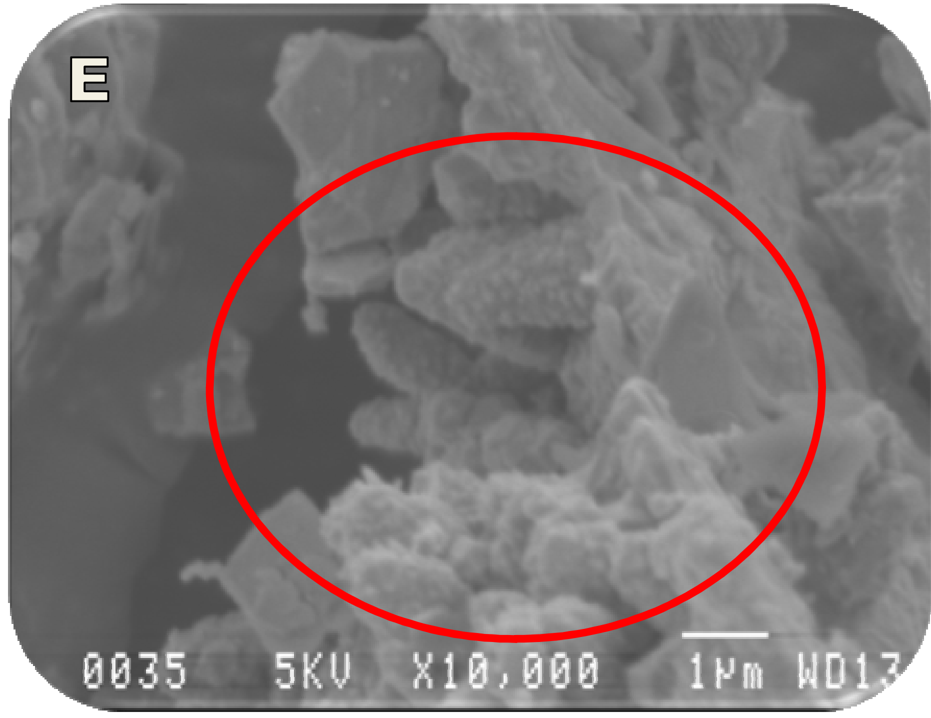
E
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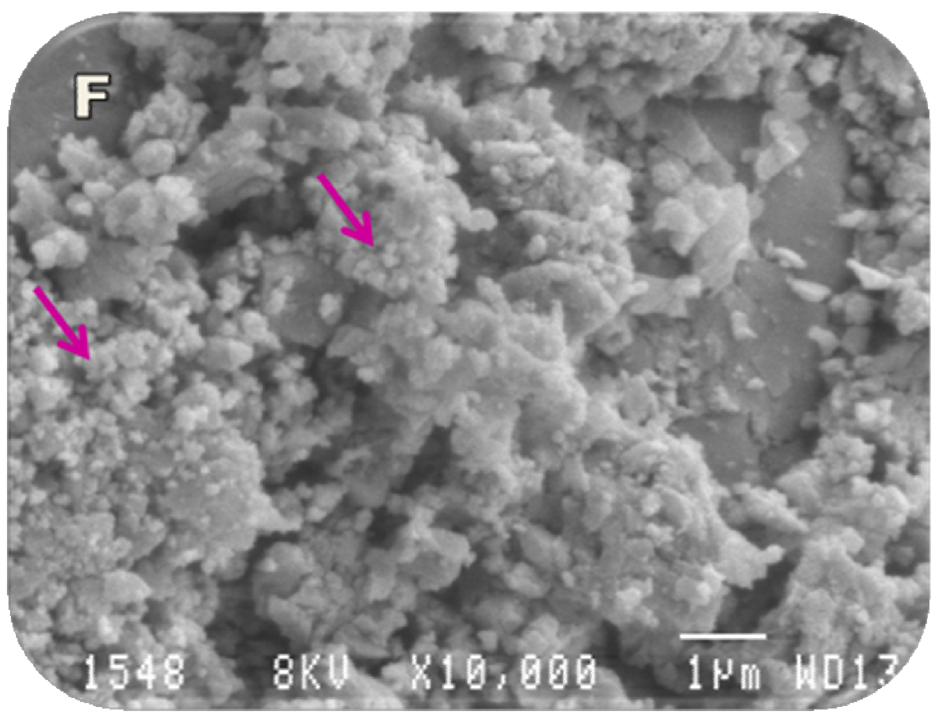
F
1548 8KV X10,000 1μm WD13

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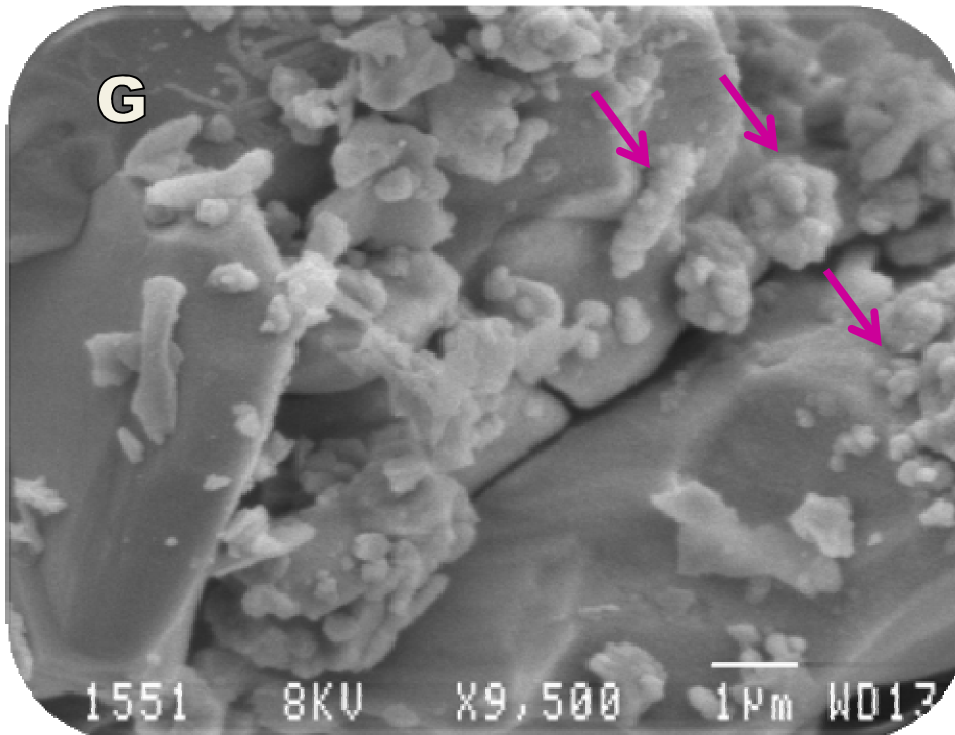

Figure 5.10: A and B represent the SEM images of isolate KU6 treatment of SK and KGT mineral types, respectively, with visible biofilm and strong attachment (arrows) to the iron surface (week 2 of the experiment). C and D represent the SEM images of isolate KU8 with high possibility of EPS secretion that binds the iron ore particles together strongly (arrows). E, F and G represents isolate SU5, KC2 and KU7, respectively, with visible forms of biofilm formation (circles and arrows).

5.4 Discussion

Unlike other metals such as copper and gold, there is no fully developed biohydrometallurgical process for treating iron ore minerals. This is partly due to the low cost of this mineral, as well as the nature of the chemical constitution (Delvasto *et al*., 2009). Biohydrometallurgical processes are mostly developed for sulfidic minerals where microbes can utilise either sulfur and iron or both as sources of energy (Rawlings, 2005). The use of such technology for biobeneficiation of Sishen iron ore may simply defeat the purpose of the technology, as iron is the major element of interest contained in iron ore materials. With all these in mind, this study has focussed on simple methods of isolating potential microbes from the iron materials and subsequently tested their potential in mobilisation of unwanted parts of the ore materials.

The choice of indigenous microbes for this investigation is essentially due to possibility of their better acclimatisation to the biobeneficiation environment. The diversity and number of bacterial isolates obtained from the iron ore surface indicates a high possibility of finding one or more potential bacteria that will be able to mobilise K or P or both from iron ore minerals. All the phylogetically identified groups, *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobateria*, isolated in this study have been previously identified and utilised for biohydrometallurgical purposes (Groudeva *et al*., 2007; Qui *et al*., 2007). At the genus level, isolates SU7, KU8, KU4, KU3 and SU3 that are closely related to *Arthrobacter*, are the dominant group. This genus of bacteria was used by Willscher and Bosecker (2003) in the leaching of alkaline siliceous slag.

Although the two iron ore minerals used in the present study are low grade, their chemical constitutions are not the same, with KGT having higher contents of the desired Fe than SK. However, KGT has higher levels of the “unwanted” elements - K and P, than SK. Such difference is also an indication that K and P contents of these minerals may be bonded and exists in different forms inside the ores. Such variation is in most cases discussed under mineralogy and was noted by Sheng *et al*. (2008) in their investigation. In that study, three different silicate minerals, namely feldspar, muscovite and biotite, contained different ratios of different compounds. This

fact can affect microbial leaching because microbes are known to utilise different mechanism to obtain nutrients from different compounds contained inside minerals (Jain and Sharma, 2004).

Bacterial screening methods used in this study were directly and indirectly adapted to acidification and direct nutrient utilisation mechanisms. According to Jain and Sharma (2004), acidification by biobeneficiation microbes can occur through production of acidic metabolites or selective preference for alkaline substrate. The former is evident in the levels of gluconic acid detected in the spent medium of all the isolates used in the shake flask experiment but it is difficult to ascertain the selective utilisation of alkaline substrate. Isolate KC1 that was included in the shake flask experiment, because of its ability to lower the pH of nutrient medium showed no such feature in the presence of the iron ore minerals. This may be due to the differences in the chemical constitution of the two media of growth (nutrient medium and modified PSM). Meanwhile, the direct nutrient utilisation was linked to the slime production by microorganisms that enhance microbial attachment to mineral surfaces and provide nutrient diffusing channels between the microbes and mineral surfaces (Gulevich *et al*; 1968; Willey *et al*., 2007).

The effects of mineral type were noticeable in all the isolates tested. The higher percentage K and P removal from SK treatments compared to KGT treatments may be related to the composition of these minerals, as previously mentioned. Although both are iron ore minerals, compositional differences in their mineral constituents could easily affect their rates of dissolution. In a study conducted on three silicate minerals (feldspar, muscovite and biotite) by Sheng *et al*. (2008), *Bacillus globisporus* Q12 strain exhibited differences in the release of K and Si from the minerals. Such differences are attributable to dissimilarities in sites of attachment for reagents such as protons and ligands involved during the bioleaching process (Huerta *et al*, 1995; Welch and Ullman, 1996). The only isolate that exhibited the ability to remove enough K up to the commercial standard was KU6, identified molecularly to be closely related to *Acinetobacter calcoaceticus*. From the available literature, no study has been able to connect this isolate to biobenefication process. However, studies have emphasized the important role of the biofilms produced by *Acinetobacter calcoaceticus* (Elkeles *et al*., 1994; Rosenberg and Ron, 1997). These are generally referred to as emulsans, highly beneficial for hydrocarbon degradation (Rosenberg

and Ron, 1997). The commercialisation of bioemulsans produced by *Acinetobacter* (Rosenberg and Ron, 1997) is an indication that further investigation of this bacterial isolate and its metabolites can provide direction in the development of a reliable method for solubilisation of iron ore minerals. It was even more interesting to discover that this isolate could reduce the P and K content of the iron ore samples (SK) below commercially approved at the same time. Such characterisitic was exhibited by strains of *Paenibacillus* spp. in a study conducted by Hu *et al.* (2006). In that study, the agricultural importance of the combined solubilising activity of the isolates was acknowledged. For the present study, it means a system that utilise only one organism can be invented for the removal of one or more impurities from iron ore minerals. Moreover, the bacterial isolate with the highest ability to mobilise P from the iron ore mineral is isolate KU8, which is closely related to *Arthrobacter* sp. This genus of bacteria have been isolated and utilised in biohydrometallurgical processes (Cardone *et al*., 1999; Luca *et al*., 2008). For instance, Cardone *et al*. (1999) utilised a species of *Arthrobacter* to remove manganese from chalcopyrite.

The mechanism involved in organic acid production by heterotrophs is essential for mobilisation of nutrients from minerals (Jain and Sharma, 2004). This study seems to have confirmed the same trend. Meanwhile, it is important to mention here that the cross-interactions that exist between the factors studied made it difficult to directly compare the organic acids with percentage K and P removal from the iron ore minerals. However, consistent detection of the organic acid in the growth medium is an indication that they are involved in the leaching process, especially gluconic acid that was detected in higher amounts compared to other organic acids (Sheng *et al*., 2008; Delvasto *et al*., 2009). One of the interesting outcomes of this study is the preferential absorption of elements by the isolates. For instance, it is apparent that isolate KU6 has ability to mobilise more K than P, whereas the reverse is the case with isolate KU8. Although this may simply be explained by the differences in their metabolic pathways, the underlying mechanism involved could be elucidated through the activities of gluconic acid. This acid could have acted in two possible ways during the iron ore solubilisation.The first is the direct activity on the surface of the mineral that involved the complexation with anions from the acid with cations of K^+, Al^{3+} (also contained in the ore) and $Fe^{3+.}$ Secondly, it is also possible for the

protons from the acid to replace interlayer K on the mineral surface due to their similar shapes and smaller size of the protons, thereby creating an "outflow" of K ions from the minerals that subsequently increased the mineral dissolution rate (Gadd, 1999; Yuan *et al*., 2004; Delvasto *et al*., 2009). Apart from the K mobilisation, such activities normally disrupt the structure of the iron ore including the phosphate content. Therefore, the solubilisation of P may be high when P is linked to the iron phase of the mineral that consists ions with high chelation constant such as Al^{3+} and Fe^{3+} contained in Sishen iron ore (Yuan *et al*., 2004; Delvasto *et al*., 2009).

Scavenging is one of the methods through which microbes obtain nutrients from minerals, but this condition is mainly created by need. Nutrient limitation is an essential condition for microbes to "re-engineer" their feeding mechanisms in order to obtain nutrients from complex substrates (Banfield *et al*., 1999; Bennett *et al*., 2001). As observed under the microscope in this study, bacterial isolates attached differently to dissimilar sites on different minerals. Different functional groups found in bacterial cell wall materials such as those detected in the present study through FTIR (carboxyl - COOH, amino - NH_2, and hydroxyl- -OH), are known to greatly influence bacterial attachment to mineral surfaces (Deo and Natarajan, 1998). The attachment is usually preceded by interactions between the bacterial cell wall and the mineral surface, a process that leads to changes in surface chemistry of the mineral (Devasia *et al*., 1993). One or more of electrostatic, hydrophobic and specific protein interactions can make this type of attachment to occur (Sampson *et al*., 2000). Scavenging is made easy for bacteria through the production of special substances that enhance their attachment to surfaces. These substances are high molecular weight compounds known as extracellular polymeric substances (EPS). They are composed of polysaccharides, proteins and nucleic acids (Omoike and Chorover, 2004). The polysaccharides part of EPS are believed to aid the component cells contained in biofilm to dissolve and utilise substrates that are normally inaccessible for utilisation (Sutherland, 2001). During scavenging, due to proximity, there could be direct utilisation of nutrients contained inside the mineral by bacteria cells of the biofilm. On the other hand, there could also be direct action of anions of organic acids from the biofilm on the mineral surface where the anions can react with cations of Fe^{3+} or Al^{3+}.

Any of the above mentioned methods would lead to an increased rate of mineral dissolution. However, as explained by Delvasto (2009), the disadvantage of this process in biobeneficiation is the accumulation of the "unwanted" elements (such as the P and K previously absorbed from the mineral ore) by the biofilm. Furthermore, leached elements could also be re-precipitated from the solution due to saturation. Such scenarios were discovered in this study when percentage quantities of K and P mobilised from the iron ore minerals were reduced between the second and third week. A feasible solution to this problem is to know the time required for the solution to be saturated with leached products, as well as monitoring the accumulation of these elements in the biofilm. This will ensure the benefication process is stopped at the appropriate time, but the idea will depend on the type of bacteria in question. For instance, two weeks could be enough for the beneficiation process involving isolate KU6 with mineral type SK.

For the first time, this study has provided information about bacterial inhabitants of Sishen iron ore. It is, however, pertinent to mention that the number of isolated bacteria from the iron ore may represent a very small fraction of the total population that inhabit the surface of this mineral. A higher number of the representative population could be obtained by utilizing molecular methods such as DGGE (Zwolinski, 2007). In addition, methods adopted in this study to screen the potential mineral solubilisers could have ommited other potential phosphate and mineral solubilisers. According to Pereze *et al*. (2007) not all phosphate-solubilisers can solubilise all forms of phosphate. In their investigation, bacteria that solubilised $Ca_3(PO_4)_2$ were unable to solubilise $FePO_4$ and $AlPO_4$. Such is the reality encountered in this study when some isolates such as KU6 and SU7 cannot solubilise most of the P contained in both minerals. Further investigation is therefore needed to investigate more types of growth media that could be used in the isolation of microbes from the minerals. In addition, from the available literature, there has not been any investigation on the use of indigenous microbes for K removal from iron ore minerals. This study has provided information and the possibilities of using indigenous microbes in this area of biohydrometallurgy. To improve on the results obtained from this study, screening methods of isolated microbes can be expanded to increase the chances of getting more and better mineral solubilisers. With this in mind, this study is therefore expected to provide a guideline for further investigations into use of indigenous microbes for biobeneficiation of iron ore minerals.

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