Identification of soil bacteria from mining environments in Rustenburg, South Africa

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Abstract: Mining industries are aware of the gainful use of bacteria in their environment. In this study two soil samples, CHRO1 and PLAT2, were collected from two mines in Rustenburg, South Africa. The detection of microorganisms from CHRO1 and PLAT2 was done by culture assay. The bacteria isolates were of various colors raging from yellow, orange, red to white and cream white, which are either rod or coccus shapes. They all stained Gram negative. Based on the API20E kit identification scheme, the isolates were identified as *Chryseobacterium indologenes, Klebsiella oxytoca, Pasteuralla pneumotropica, Enterobacter cloacae, Proteus mirabilis, Klebsiella ornothinolytica, Pseudomonas aeruginosa, Chryseobacterium meningosepticum, Chryseomonal luteola, Photobacterium clamsela, Enterobacter sakazakii, Acinotobacter baumannii, Serratia liquefaciens* and *Citrobacter koseri.*
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Introduction

Bioleaching is defined as the application of microorganisms to solubilize metals from their ores and recovering them from solution (Rohwerder et al., 2003). Microbial leaching processes are increasingly applied for metal recovery from mining and other industrial waste products that cannot be processed economically by conventional methods. Worldwide reserves of high grade ores are diminishing at an alarming rate due to the increase in demand of metals; however there exist large stockpiles of low and lean grade ores yet to be mined (Devasia and Natarajan, 2004). Mining industries have now become aware of exploiting microbial activity, thus bioleaching is no longer a promising technology but an actual economic alternative for treating specific mineral ores (Acevedo, 2002).

At moderate temperatures, the most important bacteria in bioleaching are iron and sulfur oxidizing *Acidithiobacillus ferrooxidans,* sulfur oxidizing *Acidithiobacillus thiooxidans* and *Acidithiobacillus caldus* and iron oxidizing *Leptospirillum* species (*L. ferriphilum and L. ferrooxidans*) (Coram and Rawlings 2002; Fouchera *et al*., 2003). Other major players in the bioleaching process include *Acidiphilum, Sulfobacillus*, *Ferroplasma, Metallospaera*, *Thermothrix* and *Acidianus* (Dermergasso *et al*., 1996). These bacteria share many physiological features; they are Gram negative, iron oxidizing and sulfur oxidizing chemolithoautotrophs and grow autotrophically by fixing CO_2 from the atmosphere and they also grow heterotrophically by using peptone and yeast extracts (Wei-min *et al*., 2009). Numerous studies have identified a number of potential bacterial species that are able to accumulate metals from aqueous environment. Among the bacteria, the *Bacillus* sp. are considered as those that have the high potential of metal sequestration and it has been widely used in commercial biosorption processes (Brierley *et al.,*1986). There have also been reports about biosorption of metals using *Pseudomonas* sp. *Zooglea ramigera* and *Streptomyces* sp. Other species that have been used in other research projects include *Rhodobacter sphaeroides, Alcaligenes eutrophus* and *Staphylococcus saprophyticus* (Ilhan *et al*., 2004).

Biosorption readily activate on heavy metals to detoxify the aquatic environment where the metals accumulate in the bacterial cells (Mishra *et al*., 2005). The biosorption process involves a solid phase which is called a sorbent or a biosorbent; biological matter, a liquid phase; solvent which is normally water and species to be sorbed which are called sorbates; metal ions (Das *et al*., 2008). Advantages and disadvantages of biosorption include low cost, high efficiency, minimization of chemical and biological sludge, regeneration of biosorbent and possibility of metal recovery (Kratchovil and Volesky, 1998). Disadvantages are; early saturation, this happens when the metal's interactive sites are occupied thus metal desorption has to occur. Genetic engineering of cells is limited, this is mainly because cells are not

metabolizing, and this is especially true for those bacteria that adsorb passively, so improvement of biological processes is restricted. Another thing is that there is no potential for biologically altering the metal valency state (Ahlowalia and Goyal, 2007).

Tracing bioleaching through history; development of this technology advanced rapidly during the 1980's leading to the establishment of the first commercial tank bioleaching plant at the Fairview Gold Mine near Barberton in South Africa (Acevedo, 2002). The leaching of metal, particularly copper from its ore (bioleaching) and the precipitation of copper from solution (bioaccumulation) is an ancient technology which the Chinese practiced as far back as 100-200BC and possibly even earlier (Needham and Gwei-Djen, 1974). However metal solubilization using specific microorganisms was not practiced until the 1940's but since then research contributions have helped to clarify the mechanism behind the process (Mishra, 2005). Biooxidation of sulfide ores for copper recovery has been practiced for centuries in Spain, Sweden, Germany as well as China and elsewhere (Ehrlich, 2001); however the Rio Tinto cannot be excluded in the bioleaching discussion because it is considered as the cradle of biohydrometallurgy (Mishra, 2005). The Rio Tinto mines in the south-western Spain have been exploited since the pre-Roman times for their copper, gold and silver (Lugaski, 1997). The use of bioleaching at these mines began in the 1980's where heaps of low grade copper ore were built and left for natural decomposition for about 1-3 years (Salkied, 1987). Although industrial leaching operations were conducted at the Rio Tinto mines for several decades, the contribution of bacteria to metal solubilization was not confirmed until 1961 that is when *Thiobacillus ferrooxidans* was discoveredin leacheate solutions (Salkied, 1987). Commercial application of biohydrometallurgy, designed to facilitate the activity of microorganisms, was initiated in 1980 for copper leaching from heaps, and ever since then numerous copper bioleach operations have been set up since 1980 (Brierley and Brierley 2001). An example may include the Lo Aguirre mine in Chile where it had produced about 16000 tons of ore per day between 1980 and 1996 using bioleaching (Bustos *et al.* 1993).

Today, dump/ heap leaching still remains as the most cost effective method for extracting metals from their ores which cannot be economically extracted using traditional methods and hence recently heap leaching is the most preferred method. Another example is the Quebrada in Northern Chile which can process 17300 tones of sulfide ore per day (Bustos *et al.* 1993).

To date, there are nine operating mines in South Africa, Ghana, Australia and Peru (Gold Fields, 2010). Potential benefits for bioleaching are that metals can be recovered from ores that may be considered as 'waste' which are unacceptable to smelting. There are no noxious gases that are released. It requires simple technology in terms of equipment and conditions of operation at ambient pressure and temperature; this manly applies in heap and dump leaching (Bac-Tech Mining Operation). Conventional methods of extracting metals such as smelting generate a lot of SO_2 in the environment (Stott *et al.*, 2000) and thus bioleaching a more environmentally friendly than many traditional extraction methods (Bo Fu *et al.*, 2008). At times, it is these metals that find their way into water bodies, and hazardous characteristics of the pollutants cause renal dysfunction, bone degeneration, liver, lungs and blood damage (Ebdon *et al.*, 2001). For example; cadmium is the most dangerous metal for human health due to its non-biodegradability. It is known to bind with essential respiratory enzymes (Nies, 2003) and inhibits DNA repair (Jin *et al.*, 2003). The heavy metals are non-biodegradable pollutants and tend to accumulate in living organisms (Kobya et al., 2005). The presence of such metals in aquatic environments causes severe damage to aquatic life and killing microorganisms during biological water purification process (Vinodhini and Narayan, 2008).

With the development of many industries-mining, surface finishing, energy and fuel production, fertilizer, pesticides, metallurgy, metal and steel, electroplating, electrolysis, electro-osmosis, leather, photography, electric appliance manufacturing, metal surface treating, aerospace and atomic energy installations, wastes containing metals increasingly become a threat to the environment and to humans (Wang 2002). Algae, bacteria, fungi and yeasts have proved to be potential biosorbents and can reduce the amount of metal ions in solution (Volesky, 1986). This work describes isolation of bacteria involved in bioleaching and biosorption processes and also identify the family, genus and eventually species to which the bacteria belong to.

Materials and methods

Study area and Soil Collection

Two soil samples were collected from two mines in Rustenburg South Africa which is 161.96 km from Mafikeng. Using sterile techniques, they were transported to the North-West University's Microbiology Research laboratory for analysis. Soil

sample from the first mine was named CHRO1 and from the second mine, PLAT2.

Isolation of bacteria

Upon arrival, test soil (1 g) was weighed and dissolved in 9 ml of water and stirred for a while, in order to loosen bacteria that might have attached to soil particles. A-100 µl aliquot from the ten-fold serial dilutions were spread onto Nutrient agar plates. They were incubated at 37^{O}C for 24 h. To obtain pure cultures, colonies were streaked on to fresh agar plates and incubated at the same conditions as the original colonies. The isolates were Gram stained using the standard methods.

Biochemical tests

Because bacteria are so similar in morphology, biochemical tests were used to identify them after preliminary examination of their morphology, motility and growth responses. The Oxidase test (Pro-lab Diagnostics) was performed to test the presence of a cytochrome c enzyme; by smearing the bacteria on a white paper and the formation of a purple color indicates a positive test while no color is identified as negative.

The Catalase test was done, using hydrogen peroxide H_2O_2 (Merck). This is to test if the enzyme catalase is present in the bacteria; the presence of bubbles indicates that the enzyme is present. Triple sugar iron (TSI) agar (Biolab, Merck, S.A) was used to determine the ability of the isolates to utilize 3 sugars; glucose, sucrose and lactose as the source of energy. The agar was prepared, autoclaved and poured into McCartney bottles which were placed in a slanting position to create a butt and a slant. The isolates were then inoculated onto the agar; the results were read and recorded based on color change from red to yellow, gas production and H_2S production as determined by Forbes and Weissfeld (1998). If the color of the agar remained red, it indicated that there was no reaction.

The citrate test is based on the ability of bacteria to convert citrate into oxaloacetate. Simmon's citrate agar was also prepared, autoclaved and distributed into McCartney bottles which were also placed in a slanting position. The results were read and recorded on a color change from green to blue. The blue color indicates a positive result meaning that the bacteria can use nitrate as a carbon source while green means a negative result.

The motility test was also performed; it was done by preparing a bacterial smear on a slide (B&G-Germany) covered with a cover-slip, observed under a light microscope by decreasing the light intensity. Motility was confirmed by movement of bacteria in the smear.

Confirmatory Biochemical Test -Analytical Profile Index (API 20E)

API 20E is a standard test kit that is designed for the identification of bacteria that belong to the family *Enterobacteriaceae* and other fastidious Gram negative bacteria. The test was carried out according to the instructions of the manufacturer (BioMériux, France). The results were read after incubation based on color changes of the dry substrates.

Results and Discussion

From the laboratory-based experiments, it was found that the maximum temperature at which the isolates can survive in is 37^{O}C for 48 h, even after re-streaking multiple times onto fresh nutrient agar. From this finding, one can conclude that they are mesophiles. Mesophiles are defined as organisms showing a growth temperature optimum between 25-40^{O}C (Madigan *et al.,* 2009). So, all the isolates from this work fall under this description. Observed under the light microscope, were isolates that had a thick matrix surrounding the bacteria, these isolates were identified as *Pseudomonas aeruginosa.* There have also been reports about biosorption of metals using *Pseudomonas* sp. (Ilhan *et al*., 2004).

Pseudomonas have very simple nutritional requirement and grow chemoorganotrophically at neutral pH (Madigan *et al*., 2009) as also established in this work (Table 1). They are Gram negative, no gas formed when glucose is fermented and oxidase positive (which help in distinguishing them from enteric bacteria) and finally they are motile with a help of a flagella; single or multiple (Madigan *et al*., 2009). These characteristics agree with the biochemical tests that help distinguish the isolates from each other (Tables 2, 3, and 4). The bacteria isolated and identified in this work are *Chryseobacterium indologenes, Klebsiella oxytoca, Pasteuralla pneumotropica, Enterobacter cloacae, Proteus mirabilis, Klebsiella ornothinolytica, Pseudomonas aeruginosa, Chryseobacterium meningosepticum, Chryseomonal luteola, Photobacterium clamsela, Enterobactersakazakii, Acinotobacter baumannii, Serratia liquefaciens,* and *Citrobacter koseri.* It is clear that the isolates found in this study are metal resistant. Previous research has indicated that heavy metal resistance of *P. aeruginosa* can be used to exploit for cleaning up industrial wastewater and bioremediation of heavy metal contaminated soil (Raja and Selvam 2009).

Table 1. Characteristics of isolates from samples CHRO1 and PLAT2

Sample		Shape of isolate	Color of isolate	Gram stain	Triple Sugar Iron Test Agar(TSI)			Citrate Test	Oxidase test	Catalase test	Identified Isolate
					Y	R	H_2S				
CHRO1	WBCN2	rod	white	-	-	+	-	+	-	+	*Proteus mirabilis*
	CWCN3(2)	coccus	cream white	-	-	+	-	+	+	+	*Pho. damsela*
	CWCN3(3)	coccus	cream white	-	-	+	-	+	+	+	*Pseu. aeruginosa*
	OCN3(1)	coccus	orange	-	+	-	-	-	+	+	*Acino. baumannii*
	OCN3(2)	coccus	orange	-	-	+	+	+	-	+	*Chryseom. luteola*
	CWCN5(4)	rod	cream white	-	+	-	+	+	-	+	*K. ornithinolytica*
	YCN5(1)	coccus	yellow	-	+	-	+	+	-	+	*Chryseob. meningosepticum*
	CWCN7(1)	coccus	cream white	-	-	+	-	+	-	+	*Ent. cloacae*
	CWCN7(3)	rod	cream white	-	+	-	-	+	+	+	*Ent. sakazii*
	CWCN8(6)	rod	cream white	-	+	-	-	+	-	+	*K. oxytoca*
	OSN8(1)	rod	orange	-	+	-	-	+	-	+	*Citro. koseri*
	OSN8(2)	rod	orange	-	+	-	-	+	-	+	*K. pneumonia*
	CWCSN9(2)	rod	cream white	-	+	-	-	+	-	+	*Pantoea spp.*
	CWCN10(4)	coccus	cream white	-	+	-	-	+	-	+	*S. liquefaciens*
PLAT2											
	CWCN2(1)	coccus	cream white	-	+	-	-	+	-	+	*Ent. cloacae*
	CWCN2(4)	rod	cream white	-	-	-	+	+	+	+	*Pseu. aeruginosa*
	OCSN2(3)	coccus	orange	-	+	-	-	+	-	+	*Chryseob. indologenes*
	OCSN2(4)	coccus	orange	-	+	-	+	+	+	+	*P. pneumotropica*
	CWCN4(4)	rod	cream white	-	-	+	-	+	+	+	*K. pneumonia*
	WCN4(2)	rod	white	-	+	-	-	+	-	+	*K. oxytoca*
	YCN4(2)	rod	yellow	-	-	+	-	+	+	+	*Pseu. aeruginosa*
	YCN5(2)	rod	yellow	-	+	-	+	+	-	+	*Pantoea spp.*
	YCN7(1)	rod	yellow	-	+	-	-	+	-	+	*Pantoea spp.*
	RN7(1)	cocci	red	-	-	+	-	+	-	+	*Ent. cloacae*

Table 2. Enzymes utilization tests as revealed by API20E for samples CHRO1 and PLAT2

Sample	Species	2-Nitrophenyl-βD-galactopyranoside	L-arginine	L-lysine	L-ornithine	Trisodium citrate	Sodium thiosulfate	Urease	Tryptophan deaminase	Indole production	Sodium pyruvate	Gelatin
CHRO1												
	Ent. cloacae	-	+	-	+	+	-	-	+	-	+	-
	K. pneumonia	+	+	+	-	+	-	-	-	+	-	-
	Pro. mirabilis	-	+	-	-	+	-	+	+	-	-	+
	K. ornithinolytica	+	+	+	+	+	+	+	-	-	+	-
	Pseu. aeruginosa	-	+	-	-	+	-	-	+	-	-	-
	Chryseobac. meningosepticum	+	-	-	-	+	+	-	-	-	-	+
	Chryseom. luteola	+	+	-	-	+	+	-	-	-	-	-
	Pho. damsel	+	+	+	-	+	-	-	-	-	-	-
	Pantoae spp.	+	+	-	-	+	-	-	-	+	-	-
	Ent. sakazii	+	+	-	+	+	-	-	+	-	+	+

	K. oxytoca	+	-	+	-	+	-	+	+	+	+	-
	S. liquefaciens	-	+	+	+	+	-	-	-	-	-	-
	A. baumannii	-	-	-	-	+	-	-	+	-	-	+
	Citro. koseri	+	-	-	+	-	-	-	+	+	-	-
PLAT2												
	Chryseob. indologenes	-	-	-	-	+	-	-	-	-	-	+
	K. oxytoca	+	-	+	-	+	-	+	+	+	+	-
	P. pneumotropica	+	-	-	-	+	-	+	-	-	-	-
	Ent. cloacae	-	+	+	+	+	-	+	-	-	+	-
	K. pneumonia	+	-	+	-	+	-	+	-	-	-	-
	Pantoea spp.	-	-	-	-	+	-	-	+	-	-	-
	Pseu. aeruginosa	-	+	-	-	+	-	-	-	-	+	-

-, negative; + positive

Table 3. Fermentation/Oxidation reactions as revealed by API20E for samples CHRO1 and PLAT2

Sample	Species	D-glucose	D-mannitol	Inositol	D-sorbitol	L-rhamnose	D-sucrose	D-melibiose	Amydalin	L-arabinose
CHRO1										
	Ent. cloacae	+	+	-	+	+	+	+	+	+
	K. pneumonia	+	-	-	+	+	+	+	+	+
	Pro. mirabilis		-	-	-	-	-	-	-	-
	K. ornithinolytica		+	+	+	+	+	+	+	+
	Pseu. aeruginosa		-	-	-	-	-	+	-	-
	Chryseob. meningosepticum		-	-	-	-	-	-	-	-
	Chryseom. luteola	-	-	-	-	-	-	-	-	-
	Pho. damsel	+	-	-	-	-	-	+	-	-
	Pantoae spp.	+	+	-	-	+	-	+	+	-
	Ent. sakazii	+	+	-	+	+	+	+	+	+
	K. oxytoca	+	+	+	+	+	+	+	+	+
	S. liquefaciens	+	+	+	-	+	+	+	+	+
	Acino. baumannii	-	-	-	-	-	+	-	+	+
	Citro. koseri	+	+	-	+	+	+	+	+	+
PLAT2										
	Chryseob. indologenes	+	-	-	-	-	-	-	-	-
	K. oxytoca	+	+	+	+	+	+	+	+	+
	P. pneumotropica	+	-	-	-	-	-	-	-	-
	Ent. cloacae	+	+	-	+	+	+	+	-	-
	K. pneumonia	+	+	+	+	+	+	+	+	+
	Pantoea spp	+	-	-	-	-	+	+	+	+
	Pseu. aeruginosa	+	+	+	+	+	+	+	+	+

-,negative; +positive

Table 4. Additional biochemical test as revealed by API20E for samples CHRO1 and PLAT2

Sample	Species	Oxidase	NO_2 production	Glucose fermentation	Glucose oxidation	N_2 reduction	Growth on McConkey agar	Mobility
CHRO1								
	Ent. cloacae	-	+	+	+	+	+	+
	K. pneumonia	-	+	+	+	+	+	-
	Pro. mirabilis	-	+	+	+	+	-	-
	K. ornithinolytica	-	+	+	+	+	+	-
	Pseu. aeruginosa	+	+	+	+	+	+	+
	Chryseob. meningosepticum	-	+	+	-	+	-	-
	Chryseom. luteola	-	+	+	-	+	-	-
	Pho. damsel	+	+	+	+	+	+	-
	Pantoae spp.	-	+	+	+	+	+	-
	Ent. sakazii	+	+	+	+	+	+	+
	K. oxytoca	-	+	+	+	+	+	-
	S. liquefaciens	-	+	+	+	+	+	-
	Acino. baumannii	+	+	+	+	+	+	+
	Citro. koseri	-	+	+	+	+	+	-
PLAT2								
	Chryseob. indologenes	-	+	+	+	+	-	-
	K. oxytoca	-	+	+	+	+	+	-
	P. pneumotropica	+	+	+	+	+	-	-
	Ent. cloacae	+	+	+	+	+	+	+
	K. pneumonia	+	-	+	+	-	+	+
	Pantoea spp1	-	+	+	+	+	-	-
	Pseu. aeruginosa	+	+	+	+	+	+	+

-,negative; +, positive

The need to remove Cadmium (II) {Cd (II)} is gaining wide interest from both environmental and economical viewpoints, due to its serious impacts on humans, animals and plants. When it rains the diverse components from mining industries are likely to disperse; those metals that find their ways into water may constitute sources of Cd(II) in such environment.

Besides, past research reports, determine the potential of *Citrobacter koseri* for removal of Cadmium (II)-Cd (II) from an aqueous solution through sorption (Hasan *et al*., 2008). According to the World Health Organization (WHO, 2010), the metals that are of concern include cadmium, chromium, cobalt, copper, lead, nickel, mercury and zinc. They have consequences on humans' health such as brain damage, reproductive failures, nervous system failures and tumor formation (Hamman, 2004; Mahvi, 2008). In humans Cd(II) causes itai-tai, pulmonary fibrosis, hypertension, nephrotoxicity and cancer (Hasan *et al*., 2008).. Conventional techniques for removing dissolved heavy metals include chemical precipitation, carbon adsorption, electrolytic recovery, ion-exchange, chelation and solvent extraction or liquid membrane separation (Vasudevan *et al*., 2003; Lodeiro *et al*., 2005). These methods exhibit several disadvantages such as high cost, incomplete removal, low selectivity, high energy consumption (Panjeshani and Ataei, 2008) and generation of toxic slurries that are difficult to be eliminated (Celaya *et al*., 2000; Okafor and Opuene, 2007).

In current news, Johannesburg is faced with issues of acidic water rising and contaminating water systems in the city. The acid water is currently about 600m below the city surface, but rising at a rate of between 0.6 and 0.9 m a day (Mail and Guardian, 2010). Acid water is formed underground when old tunnels fill up, the water then oxidizes with the sulfide mineral iron pyrite. The water then fills the mine and starts to spread in the environment. Speaking from a briefing, activist Mariette Liefferink, from the Federation for a Sustainable Environment, said that this poses an enormous threat, which could become worse if remedial actions are further delayed. It can have catastrophic consequences for the Johannesburg Central Business District if not stopped

in time (Mail and Guardian 2010).This is also a threat to Gauteng's poorer communities were living alongside, and in some cases on top of land contaminated by mining activities. They are exposed to high concentrations of cobalt, zinc, arsenic and cadmium as well as high levels of radioactive uranium. This leads to water supplies being in danger, because there have been some reports that heavy polluted streams drained into the Vaal River which could pose a threat to the region's water supply (Mail and Guardian, 2010). So, isolates from this work have the potential of solving this problem, but further analysis need to be done in proving this.

The research design was not intended to be bias to the identification of bacteria involved in biosorption; instead it was designed to identify bacteria found in both processes (biosorption and bioleaching). Firstly, time and budget limitations made it impractical to grow and isolate bacteria found in bioleaching processes due to the fact that the isolation is tedious and time consuming. Secondly, the agar medium that was used was a non-selective one, nutrient agar. Isolation of bacteria found in bioleaching processes requires selective bacteria such as Starkey, 9K, Ferrous Tryptone Soy Broth, Washed Agarose/ Yeast Extracts and these were not used because of budget constraint. Temperature was another limitation. Only mesophiles were identified due to the presence of only one incubator in the laboratory that operates at a single temperature of 37^0C, therefore thermophiles could not be identified and these are useful in bioleaching; as it is a process that is affective at higher temperatures not denying the fact that mesophiles are also present at temperatures suitable only for them.

It is recommended that mines should avoid allowing acid mine drainage to infiltrate the ground by using impermeable bases where heaps of dumps of ores are placed. It is also recommended that mines should obtain a closure certificate before shutting down because this is also one of the reasons that lead to AMD production in the surrounding environment. The certificate is obtainable from the Department of Minerals and Energy and the Department of Water and Environmental Affairs (Resource, 2010).

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