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Isolation of Metal Tolerant Bacteria from Polluted Wastewater

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ABSTRACT

Screening of standing and flowing water sample from Malaysian gold mine environment yielded 24 single colonies and all isolates were assessed for their metal tolerance capability. A preliminary screening on Chloride Free Medium (CFM) agar plate supplemented with 5mM of Cu^{2+}, Ag^{+} and Zn^{2+} showed that two isolates were tolerant towards Cu^{2+} ion, while two other isolates were tolerant towards Zn^{2+} ion and one single isolate was tolerant towards Ag^{+} ion. Partial identification by 16S rRNA determined that they are only two distinct species of bacteria, namely, *Bacillus* sp. and *Achromobacter* sp. The identification was supported by physical and biochemical characterizations which showed that *Bacillus* sp. was a positive rod while *Achromobacter* sp. was a gram negative coccus. Maximum tolerance concentrations (MTC) of *Bacillus* sp. and *Achromobacter* sp. were determined in liquid CFM medium and the results showed that *Bacillus* sp. could tolerate up to 20 µM Cu^{2+} ion and 2.5 mM Zn^{2+} ion, while *Achromobacter* sp. could tolerate up to 5 µM Ag^{+} and 20µM Cu^{2+} ion.

Keywords: Bacteria, Metal ion, Metal tolerant bacteria, Mining environment

INTRODUCTION

With the advancement of industrial development, environmental pollution caused by toxic heavy metals is increasingly becoming an ecological risk. Heavy metal pollution in the environment can occur naturally and it is caused by leaching of metals from soils. Effluent discharge from mining activity is another reason of accumulation of metals in water sources in Malaysia (DOE, 1997). Acid mine drainage (AMD) produced during mining activity could leach out heavy metals such as mercury, lead and arsenic from the waste ore and carried downstream as water washes over the rock surface (Corpwatch, 2007).

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This situation can cause concentration of heavy metals in mine areas to escalate up to 50 g/kg, depending on the type of metals and area of contamination (Monica, 2008).

In this work, Cu^{2+}, Zn^{2+} and Ag^{+} ions were chosen for the metal tolerance study on isolated bacteria. These three metal ions were chosen because of their differences in the toxicity level to microbial cells. Zn^{2+} ion is an essential metal ion as it serves as a micronutrient as well as a component on zinc-finger protein inside the bacteria cell (Tan, 2007; Abskharon *et al.*, 2008). On the other hand, Cu^{2+} is a non-essential metal ion where only low concentration of Cu^{2+} is needed in the cell for the activity of the enzymes to occur (Ryu *et al.*, 2003; Yu *et al.*, 2009). Both Zn^{2+} and Cu^{2+} can enhance microbial growth at low concentrations but suppress growth at high concentrations. In contrast, Ag^{+} is a toxic metal ion which can cause changes in the physiology and biochemistry of the cell at a concentration as low as 20 µM (Ratte, 1999; Slawson *et al.*, 1990).

Microorganisms are always the first biota to be contacted with metal pollution. The interaction between the microorganisms with metals has been well documented (Hughes & Poole, 1989; Slawson *et al.*, 1990). Biological organisms are easily affected directly or indirectly by heavy metal pollution. However, there are reports on the adaptation of microorganisms towards heavy metals that make them innocuous (Ibrahim, 1993; Yu *et al.*, 2009). Metal ions affect microorganism by reducing their growth and activity which can be reflected by a reduction of the growth rate and an increase in lag time (Gikas *et al.*, 2009). To survive under metal ions stress conditions, microorganisms have evolved several defence mechanisms either by quick and unspecific or slow and substrate specific (Spain & Alm, 2003). They respond to heavy metal stress using different defence systems, such as excluding metal ions from the cell, reducing to a less complex compounds, forming a complex by thiol-containing molecules and synthesizing metal binding proteins (Slawson *et al.*, 1990; Neis, 1999; Malin & Leif, 2001; Hussein *et al.*, 2004).

It is important to note that a sound knowledge of the interactions between microorganisms and metal species is fundamental to understanding the behaviour and fate of trace metals in the environment. Thus, the aims of this study were to isolate and identify indigenous bacteria from contaminated mining area and to assess their tolerance towards the toxic levels of Ag^{+}, Zn^{2+} and Cu^{2+} ions. For this purpose, bacteria were isolated from contaminated water points in the mining area. The isolates were then screened for their ability to grow on minimal media supplemented with 5mM of metal ions under investigation. The selected isolates were then characterized and identified using the biochemical and molecular biology technique. The metal tolerance capabilities of the bacteria isolates were further investigated through a maximum tolerance concentration (MTC) study. It is hoped that the results from this study can be a precursor to understanding

the interactions of microorganisms with heavy metals and will eventually lead to the development of tools for the detection of the level of metals in the environment, as well as facilitate in the *in situ* decontamination of metal-polluted waste sites.

In this study, the wastewater sample was collected from a Malaysian gold mine environment which was contaminated with approximately 5mg/l of copper and less than 1mg/l of zinc in the drainage (Natural Environment Research Council, 1995). The aim of this study was to isolate indigenous bacteria that are tolerant towards silver (Ag^{+}), zinc (Zn^{2+}) and copper (Cu^{2+}) ions that exist in the gold mine. The isolated bacteria were characterized physiologically and biochemically, as well as were tested for their degree of metal tolerance.

MATERIALS AND METHODS

Description of the Site

Penjom gold mine (4° 8' 25'' N, 101° 59' 6'' E), which is located in Peninsular Malaysia covers an area of 8.199 km^2 (2,026 acres), and was selected as a sampling location for this research. The samples were collected from different points surrounding the gold mining area. The area has a tropical climate with an annual temperature of about 20.1 to 31.8°C. Fig.1 shows the main location of the sampling site.

Fig.1: Location of the sampling site

Sample Collection

The water samples from the Malaysian gold mine environment were collected in screw-capped bottles. *In-situ* water characterizations for the pH, temperature and dissolved oxygen percentage were recorded during the wastewater collection.

Preparation of the Solutions and Media

All glassware was autoclaved prior to use. All the chemicals used were of Analar grade or equivalent and dissolved in distilled water. 100 mM of heavy metal stock solution of Zn^{2+}, Cu^{2+} and Ag^{+} were prepared by dissolving $ZnSO_4.7H_2O$, $CuSO_4.5H_20$ and $AgNO_3$ respectively in distilled water and sterilized by filtration using 0.20µm pore size (Whatman) (Sabry *et al.*, 1997; Van Nostrand *et al.*, 2007).

Bacteria Isolation and Culture Conditions

Nutrient broth (NB) was inoculated with 10% v/v water sample from the Malaysian gold mine environment and incubated at 30°C, 200 rpm. After overnight incubation, these bacterial cultures were serially diluted in distilled water (10^{-3} to 10^{-7}) before they were spread on nutrient agar (NA). The plates were incubated at 30°C for 1 day. The colonies with different morphological appearances were selected and further subcultured on the same media. All the growing bacteria cultures were stored at -80°C in 20% glycerol.

Preliminary Screening for Metal Tolerance

Each isolated culture was tested for metal tolerance by growing it on slightly modified Chloride Free Medium (CFM) agar plate (Ahmad, 1998; Ibrahim, 2003). This medium contained the following chemical reagents: Tris (4mM), $K_2HPO_4.3H_20$ (2.8mM), $KH_2PO_4.3H_20$ (2.2mM), NH_4NO_3 (18.7Mm), $CaSO_4$ (0.001Mm), K_2SO_4 (2.0mM), $MgSO_4.7H_2O$ (1.0mM) and glycerol (5g per litre). The final pH was adjusted to 7.0 to 7.4. Then, 10 ml of the following trace element solutions (pH 7-8) were added into the solution (g/l): $Na_2EDTA.2H_20$ (5.0), $Fe_2(SO_4)_3$ (0.37), $Co(NO_3)_2.6H_2O$ (0.01), ZnO (0.05), $CuSO_4.5H_20$ (0.015), $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.01) and H_3BO_3 (0.01g). Meanwhile, 10% of the agar powder (Analar grade or equivalent) was added into the agar plates. This is followed by supplementing 5mM of the investigated metal ions into the medium. Ag^{+} ion was added as $AgNO_3$, while Cu^{2+} ion was added as $CuSO_4.5H_20$ and Zn^{2+} ion was added as $ZnSO_4.7H_2O$.

Maximum Tolerance Concentration (MTC) Study

Determination of maximum tolerance towards metal ions of bacterial isolates was registered on CFM agar plates, followed by agar dilution method described by Hassan *et al*. (2008). Each agar plate was supplemented with 1-14mM Cu^{2+}, Zn^{2+} and Ag^{+} ion. The plates were inoculated with the grown isolates and incubated at 30°C for 7 days (Ibrahim, 1993).

Metal Tolerance Experiment

The improvement of the tolerance level of isolates on the increasing concentrations

of Cu^{2+}, Zn^{2+} and Ag^{+} was carried out in CFM liquid medium, followed by the serial transfer method proposed by Ibrahim (1993). The bacterial isolates that grew in the CFM liquid medium in the absence of metal ions were used as the starter cultures. This was followed by subculture into fresh CFM medium that was supplemented with 1µM of Cu^{2+}, Zn^{2+} or Ag^{+}. The cultures were incubated at 30°C, shaken at 200 rpm and the growth of the bacteria was monitored based on turbidity using Jenway spectrophotometer (wavelength: 600nm). After the bacterial culture had reached its exponential phase, it was further subcultured into the CFM liquid medium supplemented with higher concentrations of Cu^{2+}, Zn^{2+} or Ag^{+}. This procedure was repeated with increasing concentrations of metal ions until the MTC of the metal ions was reached. The bacterial cultures inoculated into CFM broth without addition of metal ions acted as a control.

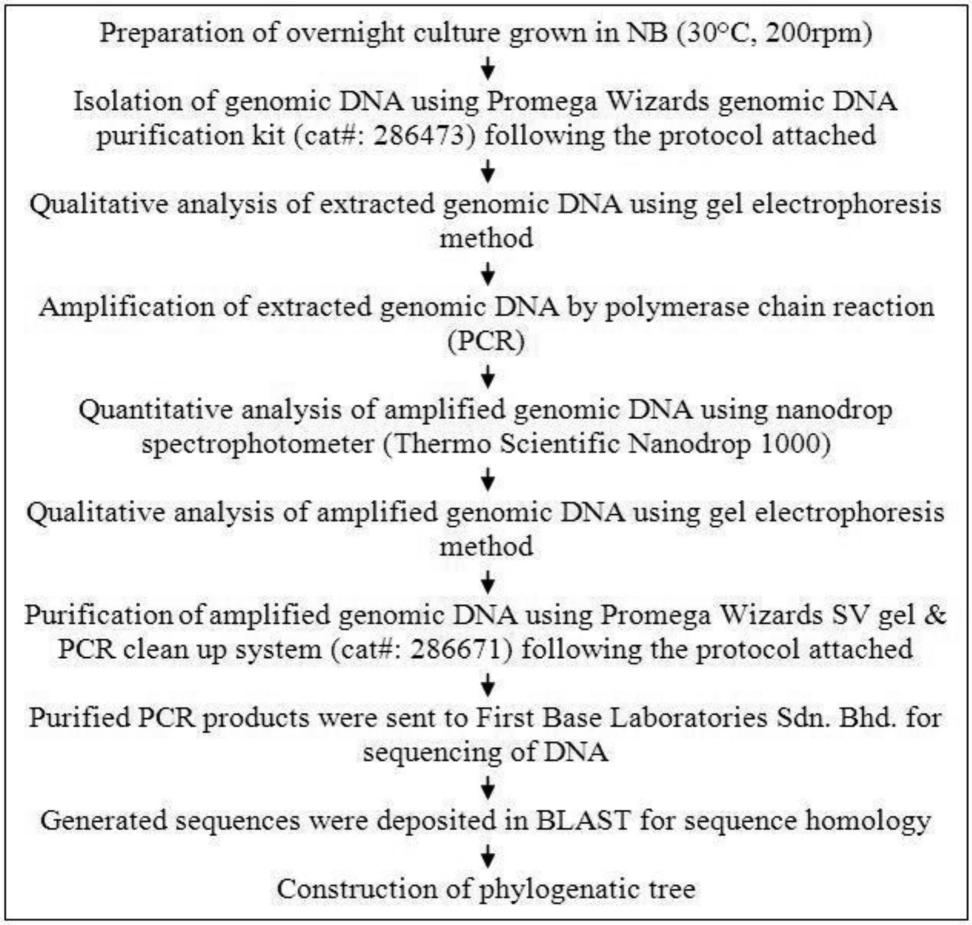

Fig.2: Partial identification using 16S rRNA method of metal tolerant bacteria

Physical Characterization of the Metal Tolerant Bacteria

The isolated bacteria were physically characterized by using a standard gram staining method to identify gram positive and gram negative bacteria (Libman *et al.*, 2006; Brown, 2007; Wan Mohd Azemin, 2010).

Biochemical Characterization of Metal Tolerant Bacteria

Further characterization of the bacterial isolates was carried out via biochemical test method, as described in the book entitled, *"Biochemical Test of Medical Bacteria"* by MacFaddin (1980).

Phylogenatic Study of Metal Tolerant Bacteria

Bacteria that showed some tolerance towards Cu^{2+}, Zn^{2+} and Ag^{+} metal ions were partially characterized using 16S rRNA. The use of 16S rRNA gene sequences is very important as a common housekeeping genetic marker because of its ability to provide bacterial identification up to species level. In this study, 16S rRNA steps were carried out following the method by Yan (2008) with slight modifications. The overall step for the 16S rRNA technique is shown in Fig.2.

An overnight culture of Cu^{2+}, Zn^{2+} and Ag^{+} tolerant bacteria in NB was prepared for the extraction of genomic DNA. The extraction of the genomic DNA was carried out using Promega Wizard DNA extraction kit following the instructions recommended by the supplier prior checked of extracted DNA on agarose gel (1% of agarose in TAE buffer) for one hour at 80mV (Ziegler *et al.*, 2007). In order to study the evolution of Cu^{2+}, Zn^{2+} and Ag^{+} tolerant bacteria, the extracted DNA gene was amplified using two sets of oligonucleotide primer. The first set was forward primers 5'-AGAGTTTGATCCTGGCTCASG-3' and reverse primer 5'-AAGGAGGTGATGCAGCC-3', while the second set was forward primer 5'- AGAGTTTGA CCTGGCTCAG-3' and reverse primer 5'-AAGGAGGTGAATCCAGC-3' using Biorad MJ mini thermocycler. The PCR reaction mixture and its condition are presented in detail in Tables 1 and 2.

TABLE 1
PCR reaction mixture

Reagents	Volume (µl)
Extracted DNA	5
Forward primer	1
Reverse primer (pH)	1
PCR master mix	25
Nucleas free water	18
Total	50

TABLE 2
PCR cycling profile

PCR steps	Temperature (°C)	Duration (min)
Initial denaturation	94	4
Denaturation (30 cycles)	94	1
Annealing (30 cycles)	50	1
Extension (30 cycles)	72	4
Final extention	72	10

The PCR products were subsequently cleaned with Promega Wizards SV gel and PCR clean up system prior to qualification

on agarose gel electrophoresis in the same conditions. 50µl of the amplified DNA, with an approximate concentration of 4ng/µl, was sent for DNA sequencing at First Base Laboratories Sdn. Bhd. All the generated sequences were deposited in the GeneBank database through BLAST (Basic Local Alignment Search Tool) which could be accessed at www.ncbi.nlm.nih.gov/BLAST for sequence homology before phylogenetic tree construction. Multiple sequence alignment was carried out using Sequence Scanner v1.0, while the construction of phylogenetic tree was performed using CLC Sequence Viewer 5.1.2 by making use of the generated sequence using bootstrapping and neighbour-joining methods.

RESULTS AND DISCUSSION

Physico-chemical Properties of the Sampling Sites

Mining-based environment is one of the sources of metal pollution into the environment. The temperature of the Malaysian gold mine environment was measured to be approximately 30.37°C, while the pH was around 7.76 with a dissolved oxygen percentage value of 5.5%. This information is important as it gives data on the most suitable parameters, primarily the temperature and pH to be used for the growth of bacterial cultures in a laboratory setting.

Isolation of the Indigenous Bacteria from the Malaysian Gold Mine Environment

The estimation of the total bacterial population present in the water sample was found to range from 200- 400 colonies/100 ml at different sampling points. A total of 24 single colonies were isolated and preserved in 20% glycerol and stored at -80°C for further studies. Table 3 lists out the number of the isolated bacteria from the standing and flowing water samples of the Malaysian gold mine environment.

TABLE 3
The number of isolated bacteria from the standing and flowing water samples of the Malaysian gold mine environment

Water samples	Number of bacteria
Standing water samples	1
Flowing water samples	23
Total isolated bacteria	24

Preliminary Screening of Metal Tolerant Bacteria

All the isolates were tested for heavy metal ion tolerance on 15 ml of CFM agar plate supplemented with 5mM of Cu^{2+}, Zn^{2+} and Ag^{+} metal ions. Ag^{+} ion was added as $AgNO_3$, while Cu^{2+} ion was added as $CuSO_4.5H_20$, and Zn^{2+} ion was added as $ZnSO_4.7H_2O$. The number of the grown bacteria and the percentage of the tolerated bacteria to Cu^{2+}, Zn^{2+} and Ag^{+} metal ions are shown in Table 4. The results showed that two isolates were tolerant towards Cu^{2+} ion, while two other isolates were shown to be tolerant towards Zn^{2+} ion, and one isolate was tolerant towards Ag^{+} ion. The concentration of 5mM metal ions is considered high for screening purpose so as to identify the bacteria that can tolerate metal ions. In her research, Ibrahim (1993) supplemented only 5- 200µM of Ag^{+} ion

to screen *Pseudomonas diminuta* and *Aeromonas hydropresearcherhila*. In another study, Piotrowska-Seget *et al.* (2005) used up to 5mM Cu^{2+} and Zn^{2+} ion as well as 0.5mM Ag^{+} ion amended in nutrient agar to count metal tolerant population whereas the used of rich medium might contribute to the growth of bacteria. In this study, the used of minimal medium supplemented with metal ions gave the maximum bioavailability of metal ions to the bacteria culture.

TABLE 4
The number of isolates growing on CFM agar medium supplemented with 5mM of Cu^{2+}, Zn^{2+} and Ag^{+} metal ions

Metal ion	Number and percentage of bacteria tolerant towards 5mM metal ion	Bacteria designation
Cu^{2+}	2* (8.3)	32E4, 32F5
Ag^{+}	2* (8.3)	22D2, 21H1
Zn^{2+}	1* (4.17)	11F1

*Values indicated the number of tolerant isolates
Values in the parentheses indicated percentage of tolerant isolates

Determination of the Maximum Tolerance Concentration (MTC) of Metal Tolerant Bacteria

The two isolates shown to have grown on the CFM agar plates supplemented with 5 mM Cu^{2+} ion were designated as 32F5 and 32E4, while two other Zn^{2+} tolerant isolates were designated as 22D2 and 21H1 and a single isolate that grew on CFM agar plate supplemented with 5mM Ag^{+} ion was designated as 11F1 (see Table 4). These five bacteria were streaked on the CFM agar plate supplemented with 1-14 mM of respective metal ions to further investigate their degrees of tolerance towards metal ions. The growth pattern on the individual isolates towards Cu^{2+}, Zn^{2+} and Ag^{+} metal ions is shown in Table 5.

The results in Table 5 show that all the bacteria grew on the CFM agar medium in the absence of metal ions. Bacteria 21H1 was found to be able to tolerate Zn^{2+} ion up to 13mM while bacteria 22D2 tolerated up to 10mM Zn^{2+} ion concentration. Meanwhile, 21H1 was shown to grow faster than 22D2 and 21H1 grew on the CFM agar

TABLE 5
The growth pattern of Cu^{2+}, Zn^{2+} and Ag^{+} tolerant isolates

Heavy metal concentration (mM)		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Zn^{2+}	22D2	+	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{6}$	$+^{7}$	$+^{7}$	$+^{7}$				
	21H1	+	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{5}$	$+^{6}$	$+^{6}$	$+^{6}$	$+^{7}$	$+^{7}$	$+^{7}$	
Cu^{2+}	32E4	+	$+^{4}$	$+^{7}$												
	32F5	+	$+^{5}$	$+^{7}$												
Ag^{+}	11F1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Subscript number showed the days needed for heavy metal bacteria to grow on the CFM agar plate added with heavy metal ion
*: Bacterial growth cannot be observed

plate supplemented with 7mM Zn^{2+} ions in 5 days and easily grew up to 13 mM concentration within 7 days. The growth of 22D2 was found to be much slower and it tolerated lower concentrations of Zn^{2+}. The highest Zn^{2+} ion concentration that it could be tolerated was 10mM. In contrast, the bacteria that grew on the CFM agar plate added with Cu^{2+} ion could grow in the Cu^{2+} ion concentration of up to 2mM. Both Cu^{2+} tolerant 32E4 and 32F5 grew slower on the CFM agar plate added with Cu^{2+} ion, in which 32E4 only started to grow on day 4, while 32F5 started to grow on day 5.

Physical and Biochemical Characterization of Metal Tolerant Bacteria

Cu^{2+} tolerant 32E4 and 32F5, Zn^{2+} tolerant 22D2, whereas 21H1 and Ag^{+} tolerant 11F1 were characterized based on the colony and cell morphology as well as the biochemical and gene analysis. The physical and biochemical characterization of the metal tolerant bacteria were determined according to the book by MacFaddin (1980) entitled, "Biochemical Tests for Identification of Medical Bacteria."

Physical Characterization of the Isolates

Colony morphology

Ag^{+} tolerant isolate, 11F1, was found to be creamy white in colour and round in shape, while the elevation and margin of the colony were entire and raised. Zn^{2+} tolerant bacteria 21H1 displayed a similar colony morphology with Ag^{+} tolerant bacteria, 11F1. Cu^{2+} tolerant bacteria, 32F5 and Zn^{2+} tolerant bacteria 22D2 shared the same colony morphology characteristics where colony colour, colony margin and colony elevation were creamy white, raised and undulate, respectively. They both differ in terms of the shape of the colony where 22D2 was wavy, and 32F5 was round. Meanwhile, Zn^{2+} tolerant bacteria (21F2) was easily distinguishable because of its colour, which is yellow. It was also found to be round in shape and acquire raised margin and entire colony elevation. Cu^{2+} tolerant bacteria (32E4) was creamy yellow in colour and round in shape, while the colony margin and colony elevation were raised and undulated. A detailed result for the colony morphology of each bacterial isolates is shown in Table 6.

TABLE 6
The Colony morphology of metal tolerant bacteria

Characteristics	Metal tolerant bacteria				
	11F1	22D2	21H1	32F5	32E4
Colony colour	Creamy White	Creamy White	Creamy White	Creamy White	Creamy Yellow
Colony shape	Round	Wavy	Round	Round	Round
Colony margin	Raised	Raised	Raised	Raised	Raised
Colony elevation	Entire	Undulate	Entire	Undulate	Undulate

Cellular morphology

Cu^{2+} tolerant 32E4 and 32F5 were found to be both gram positive bacteria and rod shaped. Ag^{+} tolerant bacteria, 11F1, was found to be a gram negative cocci, while 22D2 which was a Zn^{2+} tolerant bacteria was found to be a gram positive rod. Zn^{2+} tolerant bacteria 21H1 is a gram negative cocci. Details of the cellular morphology and Gram reaction are given in Table 7.

Biochemical Characterization of the Isolates

The biochemical characterization of metal tolerant bacteria was carried out in order to identify the bacterial isolates. In this work, the biochemical tests were carried out according to the methods by MacFaddin (1980). In this work, Mac Conkey agar was used to differentiate the gram positive and gram negative bacteria in which bile salt that contained in the agar would completely inhibit the growth of the gram positive bacteria. Zn^{2+} tolerant bacteria 22D2 and Cu^{2+} tolerant bacteria 32E4 and 32F5 were completely inhibited on the Mac Conkey agar plate. This result confirmed that 22D2, 32E4 and 32F5 were gram positive bacteria. Nevertheless, Ag^{+} tolerant bacteria and Zn^{2+} tolerant bacteria 21H1 did not grow on Mac Conkey agar plate and this indicated that they were gram negative bacteria. Lactose fermenting bacteria could also be distinguished using Mac Conkey agar plate. Both Ag^{+} tolerant bacteria 11F1 and Zn^{2+} tolerant bacteria 21H1 turned the Mac Conkey agar plate to yellow and this showed that they were non-lactose fermenting bacteria. All the metal tolerant bacteria were found to be motile where they migrated from the stab point to diffuse into the medium and caused turbidity. The Oxidation-fermentation test (OF test) was performed using commercial oxygen-fermentation medium (Difco). The Ag^{+} tolerant bacteria 11F1 and Zn^{2+} tolerant bacteria 21H1 were found to be able to metabolize a carbohydrate under aerobic condition. In contrast, Zn^{2+} tolerant bacteria 22D2 and Cu^{2+} tolerant bacteria, 32F5 and 32E4 were fermentative bacteria which could utilize carbohydrate in the absence of oxygen. The oxygen requirement test showed that all the metal tolerant bacteria were obligate aerobes which strictly need oxygen to grow. Commercial Christensen urease agar was used to identify the bacteria with the ability to split urea into ammonia. Ag^{+} tolerant bacteria 11F1 and Zn^{2+} tolerant bacteria 22D2 were found to have the ability

TABLE 7
The Cellular morphology of metal tolerant bacteria

Metal tolerant bacteria					
Characteristics					
	11F1	22D2	21H1	32F5	32E4
Gram stain test	Negative	Positive	Negative	Positive	Positive
Cell morphology	Cocci	Rod	Cocci	Rod	Rod

TABLE 8
The Biochemical characteristics of metal tolerant bacteria

Characteristics	Metal tolerant bacteria				
	11F1	22D2	21H1	32F5	32E4
Mac Conkey agar plate test	+	-	+	-	-
Lactose ferment test	-	*	-	*	*
Motility test	Motile	Motile	Motile	Motile	Motile
Oxidation- Fermentation test	O	F	O	F	F
Oxygen requirement test	Obligate aerobes	Obligate aerobes	Obligate aerobes	Obligate aerobes	Obligate aerobes
Christensen urease test	+	+	-	-	-

*No growth observed

to produce two molecules of ammonia from urea compound. Table 8 shows detailed results of the biochemical characterization of metal tolerant bacteria.

However, the results of the biochemical tests that were carried out in this work were not sufficient enough to identify the isolates accurately. Thus, 16SrRNA analysis was performed as it gives a better degree of accuracy for bacterial identification.

16S rRNA Gene Sequence Analysis

The gene sequence analysis was performed to further identify the bacteria and to support the results from the biochemical analysis. Fig.3 and Fig.4 show details of the constructed phylogenatic tree. The phylogenatic analysis of 11F1 and 21H1 showed that they are closely related (88% of bootstrap replication) to *Achromobacter piechaudii* strain Shan11. originated from *Alcaligenaceae bacterium* JS 8 (89% of bootstrap replication). On the other hand, the gene sequence analysis of Cu^{2+} tolerant bacteria 32E4 and 32F5 and Zn^{2+} tolerant bacteria 22D2 showed that they are from *Bacillus* genus and shared 100% of bootstrap replication with *Bacillus anthracis* strain V11DMK. Table 9 shows a summary of the bacterial species and metal ions that they could tolerate.

TABLE 9
The Identified species of $Cu^{2+,}$ Zn^{2+} and Ag^{+} tolerant bacteria

Metal tolerant bacteria	Metal ions	Identified species
11F1	Ag^{+}	*Achromobacter* sp.
22D2	Zn^{2+}	*Bacillus* sp.
21H1	Zn^{2+}	*Achromobacter* sp.
32F5	Cu^{2+}	*Bacillus* sp.

Metal Tolerance Experiment

Bacillus sp. and *Achromobacter* sp. were identified to be tolerant towards Cu^{2+}, Zn^{2+} and Ag^{+} ions. In this work, *Bacillus* sp. could tolerate Cu^{2+} ion and Zn^{2+} ion while *Achromobacter* sp. could tolerate Ag^{+} and Zn^{2+} ion. The results shown in Table 10

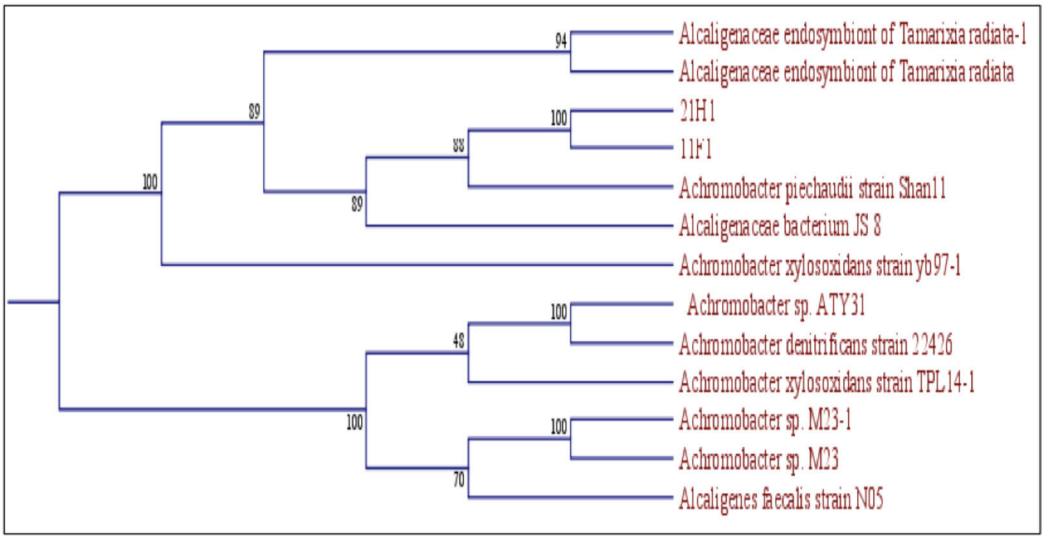

Fig.3: The Phylogenatic tree of Ag^{+} tolerant bacteria, 11F1 and Zn^{2+} tolerant bacteria 21H1

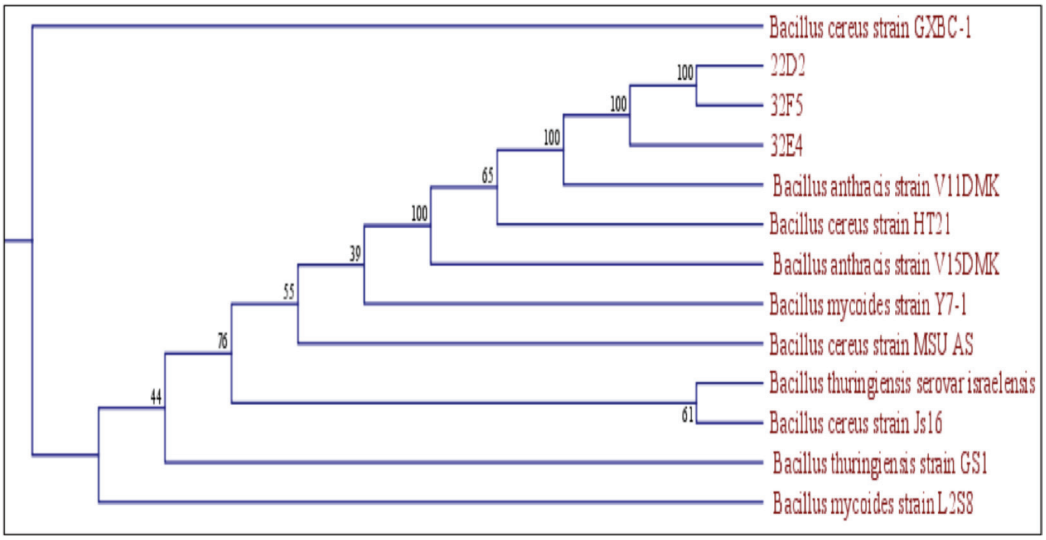

Fig.4: The phylogenatic tree of Cu^{2+} tolerant bacteria, 32E4 and 32F5 and Zn^{2+} tolerant bacteria 22D2

indicate that *Bacillus* sp. could tolerate up to 2.5mM Zn^{2+} ion and 0.02mM Cu^{2+} ion. On the other hand, *Achromobacter* sp. could tolerate 0.01mM Zn^{2+} ion and 0.005mM Ag^{+} ion. The tolerant value appeared to be lower than those of the other reports which had claimed that *Bacillus cereus* and *Bacillus thurengiensis* could tolerate 150µM and 0.2mM Cu^{2+} ion, respectively, and *Achromobacter* sp. was found to be able to tolerate Zn^{2+} ion up to 20µM (Hassen *et al.*, 1997; Raja *et al.*, 2006). However, in some previous studies, complex media was used for the metal tolerance studies; in

this study, minimal media (i.e. CFM) was used. The use of the minimal medium in this work reduced the negatively charged ion like chloride which prevents metal ion precipitation, and hence giving the maximum bioavailability of metals to the bacteria.

TABLE 10
The maximum tolerance concentrations (MTC) of *Bacillus* sp. and *Achromobacter* sp

Bacteria species	Metal ion	Maximum tolerance concentration (MTC) (mM)
Bacillus sp.	Cu^{2+}	0.02
	Zn^{2+}	2.5
Achromobacter sp.	Ag^{+}	0.005
	Zn^{2+}	0.01

Bacillus sp. and *Achromobacter* sp. grown in the CFM liquid medium in the absence of metal ions worked as a control. Fig.5 and Fig.6 show the growth curve of *Bacillus* sp. and *Achromobacter* sp. in the CFM liquid medium in the absence of metal ions. Both the bacteria showed lengthy log phase, especially *Achromobacter* sp. which took approximate 150 hours before entering lag phase whereas *Bacillus* sp. took about 30 hours. This might be due to the use of the minimal medium for growth.

CONCLUSION

A study on the isolation of bacteria from a Malaysian gold mining environment yielded 5 bacterial isolates that were later identified to be only 2 distinct species, namely, *Bacillus* sp. and *Acromobacter* sp. Zn^{2+} tolerant bacteria have the ability to tolerate up to 2.5mM Cu^{2+} ion, while Cu^{2+} tolerant bacteria could adapt up to 20µM Cu^{2+} ions. The Ag^{+} tolerant bacteria have the lowest tolerance, which is only up to 5µM of Ag^{+} ion concentration. These isolates are of

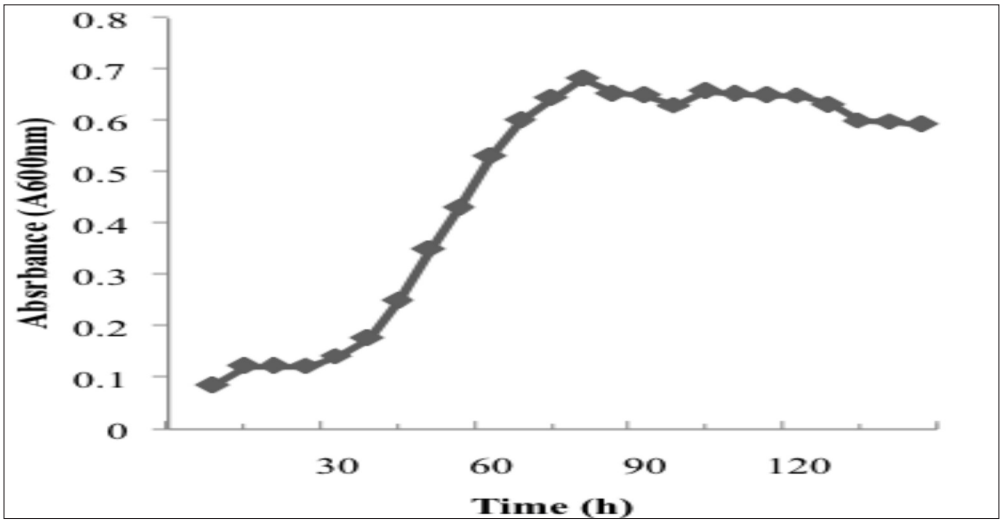

Fig.5: The growth curve of *Bacillus* sp in CFM liquid medium in the absence of metal ion

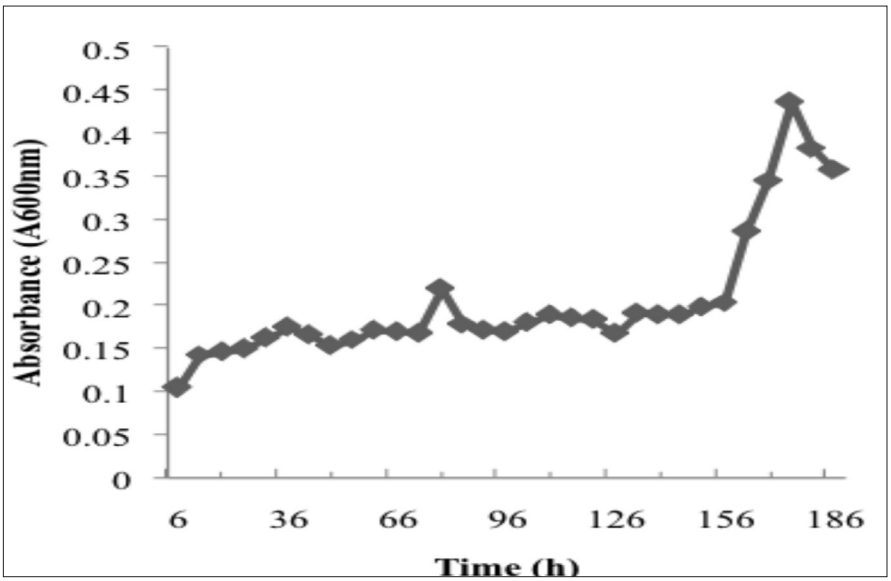

Fig.6: The growth curve of *Achromobacter* sp. in CFM liquid medium in the absence of metal ion

interest for further characterization in order to understand their mechanisms for metal tolerance and they have the potential to be developed for bioremediation of toxic heavy metals in contaminated environments.

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