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# SCREENING AND IDENTIFICATION OF HEAVY METAL RESISTANT BACTERIA (MH1) *OCHROBACTRUM* SP. ISOLATED FROM INDUSTRIAL EFFLUENT

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## ABSTRACT

The present study deals with screening and identification of heavy metal resistant bacterial strain which was isolated from heavy metal-polluted industrial effluent the species of bacteria isolated was identified as *ochrobactrum* sp. based on the 16S rRNA gene sequence analysis. The accumulation of heavy metals, and their detoxification by bacterial isolate provide an additional mechanism of environmental bioremediation. The bacterial strain was also evaluated for resistance from different heavy metals in different concentrations. The identified heavy metal tolerant bacterial isolate could be useful for the efficient removal of heavy metal in contaminated waste water.

**Keywords**: Isolation, bacteria, molecular, characterization, effluent.

## INTRODUCTION

Industrial developments led to use of multiple synthetic substances by manufacturing industries which result in generating wastewater that contain high levels of conventional as well as non-conventional contaminants that diminished the quality of water and ecological set up (Forster, 2003; Orta de Velasquez *et al*., 2006). The world anthropogenic discharges are said to be larger than or almost equivalent to natural discharges for the majority of the trace metals (Pacyna and Pacyna, 2001). Bioremediation is the process of using microorganisms and other biological materials e.g., Bacteria, fungi, or algae to detoxify or transform pollutants from toxic to their non-toxic forms it depend on the microbial enzymatic behavior to degrade the offending pollutants. Heavy metal ions is not easy to remove in environment that are already contaminated, contrary to many other organic pollutants, heavy metals cannot be biologically or chemically degraded and are therefore difficult to control. The end products of successful bioremediation are nontoxic and can be fit in without damage to the environment and living organisms (Atlas and Philp, 2005; Naik and Dubey, 2013) Among the inorganic contaminants, remediation approaches for heavy metals differ from those of the organic compounds due to it being non-biodegradable (Gupta and Rastogi, 2008). Hence, immobilization and physical removal have to be made. Though some heavy metals are essential for many biological processes, but become toxic at high concentrations (Abskharon *et al*., 2008) This is because of oxidative capacity to form free radicals and their ability to replace essential metals in enzymes, interrupting their normal activity (Ghosh *et al*., 2000). Some heavy metals are not essential and tend to accumulate in different organisms due to their toxicity even at lower concentrations (Naja and Volesky, 2009). Cadmium, Chromium, Mercury, lead, arsenic, copper, cobalt, are among the most toxic heavy metals base on United States Environmental Protection Agency (EPA) priority list of pollutants. The removal of heavy metals is considered an important issue with respect to the environment and economical concerns (Ozdemir *et al*., 2012). There are several methods for the removal of heavy metals from contaminated environment such as, chemical precipitation, adsorption on activated carbon, reverse osmosis, ion exchange, electro-dialysis and ultrafiltration (Huang *et al*., 2010; Kadirvelu *et al*., 2001). Nevertheless, the feasibility of economic and technical constraints might hinder the successful application of these methods (Hamouda *et al*., 2015). Bioaccumulation and biosorption are innovative technology using living or dead biomasses to remediate poisonous metals from contaminated environment (Atlas and Philp, 2005). Several microbial biomasses such as bacteria, yeast, fungi and algae were used widely for absorption of different metal ions (Krishna *et al*., 2012; Volesky, 1995). There

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are many mechanisms involve in biosorption some are not fully understood. Biosorption mechanism may be classified according to dependence on the cell's metabolism, which is called metabolism dependent or according to the location where the metal removed from solution is found which is called Non - metabolism dependent/ metabolism independent like extracellular accumulation Cell surface sorption precipitation and Intracellular accumulation (Kuyucak and Volesky, 1989; Mohamed and Abo-Amer, 2012). During metabolism independent, metal uptake is by physicochemical interaction between the metal and the functional groups present on the microbial cell surface. This is based on physical adsorption, ion exchange and chemical sorption, which is not dependent on the cells' metabolism (Ahalya *et al*., 2003). Cell walls of microbial biomass, mainly composed of polysaccharides, proteins and lipids have abundant metal binding groups such as carboxyl, sulfate, phosphate and amino groups.

## MATERIALS AND METHODS

### STERILIZATION OF GLASSWARE AND OTHER MATERIALS

All glassware used were thoroughly washed with deionized water and detergent, rinsed and allowed to dry. The glassware were then enfolded with aluminum foil and sterilized. The distilled water used for serial dilutions, was autoclaved at 121°C for 15 minutes. The workbench was cleansed with 75% alcohol prior and after every experiment.

### Growth Media and Culture Conditions

The isolated bacteria were grown at 37°C for 48 hours in sterilized nutrient broth containing in gram per liter distilled water, glucose, 1.0 g; peptone, 5.0 g; and beef extract, 3.0 g. The pH Adjusted to 6.8 ± 0.1 by the addition of either HCl/NaOH. Suspension for inoculums was obtained by growing the isolated strain MH21, in 5 ml sterilized nutrient broth, which was incubated at 37°C for 48 hours under (Nanda *et al*., 2011; Pandit *et al*., 2013).

### Sampling and bacteria analysis

The samples from the metal industry located in Klang (Malaysia) were collected in sterile glass bottles, transported on ice to the laboratory and the bacteria was isolated using a serial dilution procedure. The metal resistant bacteria were enumerated on nutrient agar and Mac-Conkey agar (Aldrich) respectively, complemented with 50mg/l concentrations of Cr, Cu, Pb and Cd by the standard pour plate technique (Srivastava *et al*., 2008). Plates were incubated at 37°C for 2 days and the total number of bacteria was determined. Further isolation of bacterial colonies was done by streaking on several nutrient agar plates for further studies.

### Colony Morphology

For partial identification of the bacteria was carried out by determining the colony color, elevation shape margin of the isolated bacteria was observed and cell morphology of the isolates were determined using microscope to characterize the isolates based on their gram reactions (Pandit *et al*., 2013)

### Growth measurements

For determination of the bacterial growth the isolates LB medium supplemented with up to 200 mg/l of various heavy metals used in the study and were kept at shacking condition at 150 RPM at 37◦C. The growth was then observed by evaluating the absorbance at 600 NM by using spectrophotometer at different interval of time from 6, 12, 18, 30, 36, 42 and 48 hours respectively. Bacterial growth was monitored by measuring the optical density of the growth cultures at 600 NM using UV/Vis spectronic spectrophotometer (Nanda, 2011; Raja *et al*., 2009).

### 16S rRNA Sequence Analysis

The potential of bacterial analysis sequence of the 16S rRNA gene has been broadly used as a taxonomic study to identify the bacterial species (He *et al*., 2010; Kommedal *et al*., 2009). Identification of bacteria using 16S rRNA gene provides better, faster and accurate results of identifying bacteria in addition to contribute important new species discovered in microbiology laboratories (Anjum *et al*., 2011; Pfeifer *et al*., 2011). The genomic DNA of the selected colonies was isolated from overnight cultures in a 30 ml sterile nutrient broth, which was incubated at 37ºC for 1 day using an orbital shaker at 150rpm the cell was then harvested by used of centrifuge at about 16000 × g for 2 minutes to pellet the cells. The supernatant was then discarded. The DNA of the isolate was extracted using DNeasy Blood and Tissue Kit following the manufacturers guide. The genomic DNA was then analyzed by 1% (w/v) Tris borate EDTA, (TBE) agarose gel electrophoresis by using a 1kb DNA ladder as a marker so as to visualize and estimate the size of the isolated DNA. Amplification of the 16S gene sequencing. Forward and reverse universal primers was used the sequence are as follows; 5’ - AGAGTTTGATCMTGGCTCAG -3'’ and 5'-TACGGYTACCTTGTTACGACTT -3' Synthesized by first base laboratory Berhad Malaysia. The polymerase chain reaction (PCR) mixture which contain 0.5 µl of 100mM forward primer, 0.5 µl of 100mM of reverse primer (First Based), 1 µl of DNA, 2 µl of Taq polymerase in $NH_4SO_4$ buffer, 2 µl of 20 mM $Mgcl_2$, 0.5 µl of 10 mM dioxynuceoside triphosphates (dNTP), 0.5 µl

of Taq polymerase (MBI ferments Lithuania) and 43 µl of sterile deionize water total volume of 50µl was used as the mixture. The purified PCR product was sequenced at First based laboratory Malaysia using the same forward and reverse primers the sequence was then matched with a known database of various 16S rRNA sequencing analysis in the composite non redundant database using a basic local alignment system known as blast 2 sequence search program. www.ncbi.nih.gov/BLAST/bl2seq.

**Phylogenetic Analysis**

Ten most closely interrelated 16S rRNA gene sequences were recovered from GenBank were compared with isolate MH1 and aligned using cluster W (Krohn *et al*., 2014; Torvinen *et al*., 2010). Phylogenetic analysis was carried out using Neighbor Joining (Unrooted Tree) by the NCBI Blast tree method. Mega software version 6.0 was used for the construction of the phylogenetic tree using the neighbor joining method.

## RESULTS AND DISCUSSION

A total number of 21 bacterial genera were recorded from the industrial wastewater. Five bacterial genera were selected and out of the 5 isolate selected were identified as two related to *Bacillus* sp. one *Brevundimonas* sp. One *ochrobactrum* sp. and *Brucella* sp. And all were able to grow in high concentrations of heavy metals. All the bacterial isolates exhibited high resistance to heavy metals with minimum inhibitory concentration (MIC) for heavy metals ranging from 50µg/ml to 200µg/ml. Isolation of bacteria specie from metal contaminated environment would represent an appropriate practice to select metal resistant bacterial strains that could be used for heavy metal removal purposes (Velasquez and Dussan, 2009).

## COLONY AND CELL MORPHOLOGY

In this study the bacterial isolate (MH1) was found to be round in shape and milky white in color, whereas the elevation and the margin of the colony were entire margin and raised. (Table 1) Also the isolate that show high tolerance to Cd, Cr, Cu and Pb. The cell of the strain was rod-like and pink in color showing features of gram negative bacteria (Fig.1).

**Growth measurements**

Biomass growth measurement revealed a typical bacterial growth pattern. With increase in incubation time the bacterial biomass development also increased. The bacterial was able to survive in various heavy metals concentrations. The growth of the bacterial isolate was found to be slower compare with the control after incubation for 48 hours.

Table. 1 The Colony Morphology of the strain MH1.

| Isolate name | MH1 |
|---|---|
| Shape | Round |
| Colony color | Milky white |
| Margin | Entire |
| Elevation | Raised |
| Cell morphology | Rod |
| Cell color | Pink |
| Gram reaction | Gram negative |

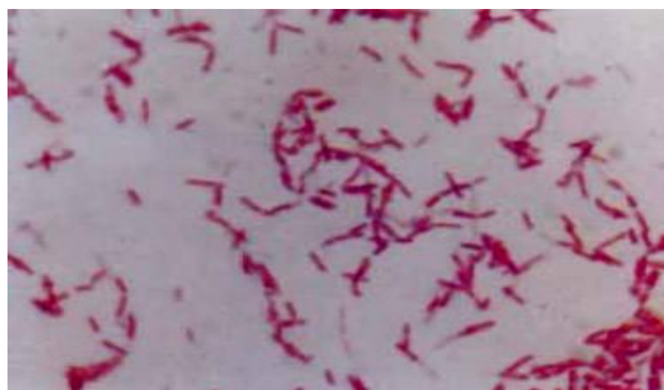

Fig. 1. Gram stain picture of isolate MH1 using Nikon microscope with 100x magnification.

Many researchers reported similar results (Abskharon *et al*., 2008; Raja *et al*., 2009; Tripathi *et al*., 2005). The better growth of bacteria in the medium containing heavy metals attributed to the capability of microorganisms to acquire many survival mechanisms to the presence of toxic substances in their environment. Pb and Cr were the best tolerated metals. While Cu and Cd were the most toxic elements for the strain MH1 (Fig. 2).

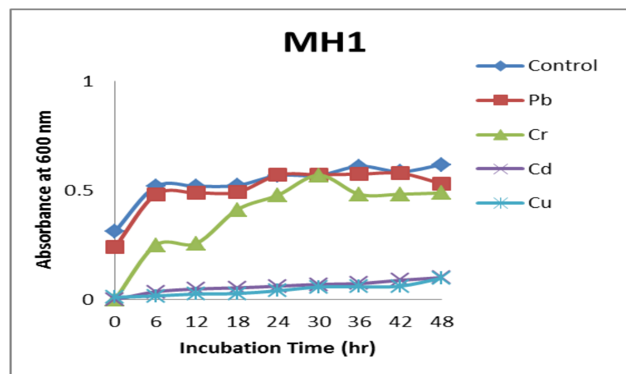


Fig. 2. Growth response of strain MH1 against heavy metals Cu, Cr, Cd and Pb concentrations.

**16S rRNA gene Sequence Analysis**

For the PCR process, the genomic DNA that was previously obtained from the DNA extraction procedure was used as a template in the amplification of the 16S rRNA gene.

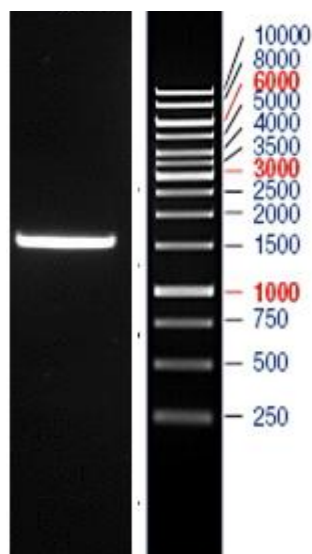


Fig. 3. The Agarose gel electrophoresis of PCR product of 16S rRNA gene of the bacterial isolates MH1.

Both the universal primer used in the amplification of the gene which was synthesized by 1st base Sdn Bdn, Malaysia. The PCR product was approximately 1 kb as shown in the Figure 3 observed using 1. % agarose gel electrophoresis and was sent for gene sequencing. The sequence differences in the hypervariable regions reveal the isolate variation.

The forward sequences and reverses complement of MH1 isolates was obtained after sequencing procedures. Those sequences were aligned for homology using the BLAST 2 sequences (www. ncbi.nlm.nih.gov /BLAST/bl2seq/). The sequence were combined at bases giving the minimum ambiguous gap. The combine 16S rRNA genomic sequence were compared with ten most closely related species in the gene bank database by use of blast sever at NBCI Figure 4.

**Phylogenetic Analysis**

Phylogenetic method has been broadly used for bacterial phylogeny, leading to the creation of large database domain and its application to identification of bacteria, which contains many clinical and environmental uncultured microbes (Scholle *et al*., 2003). Mega software version 6.0 was used for the construction of the phylogenetic tree using neighbor joining method a high bootstrap value 99% - 100% was seen when the isolates were associated with closely related strain in the NCIB database (Fig. 5) indicating that the relationship is significant.

| | Description | Max score | Total score | Query cover | E value | Ident | Accession |
|---|---|---|---|---|---|---|---|
| ✔ | Ochrobactrum lupini strain LUP21 16S ribosomal RNA gene, complete sequence | 2406 | 2406 | 100% | 0.0 | 99% | NR_042911.1 |
| ✔ | Ochrobactrum anthropi strain ATCC 49188 16S ribosomal RNA gene, complete sequence | 2401 | 2401 | 100% | 0.0 | 99% | NR_074243.1 |
| ✔ | Ochrobactrum cytisi strain ESC1 16S ribosomal RNA gene, complete sequence | 2401 | 2401 | 100% | 0.0 | 99% | NR_043184.1 |
| ✔ | Ochrobactrum anthropi strain NBRC 15819 16S ribosomal RNA gene, partial sequence | 2385 | 2385 | 99% | 0.0 | 99% | NR_113811.1 |
| ✔ | Ochrobactrum tritici strain SCII24 16S ribosomal RNA gene, partial sequence | 2383 | 2383 | 100% | 0.0 | 99% | NR_028902.1 |
| ✔ | Ochrobactrum tritici strain NBRC 102585 16S ribosomal RNA gene, partial sequence | 2367 | 2367 | 99% | 0.0 | 99% | NR_114148.1 |
| ✔ | Ochrobactrum anthropi strain LMG 3331 16S ribosomal RNA gene, partial sequence | 2358 | 2358 | 97% | 0.0 | 99% | NR_114979.1 |
| ✔ | Ochrobactrum tritici strain SCII24 16S ribosomal RNA gene, partial sequence | 2340 | 2340 | 97% | 0.0 | 99% | NR_114980.1 |
| ✔ | Brucella abortus strain 544 16S ribosomal RNA gene, partial sequence | 2318 | 2318 | 99% | 0.0 | 99% | NR_042460.1 |
| ✔ | Brucella ceti 16S ribosomal RNA, complete sequence | 2316 | 2316 | 100% | 0.0 | 98% | NR_121762.1 |

Fig. 4. Top 10 Hit Blast Results OF MH1 STRAINS against NCBI 16S ribosomal RNA sequence.

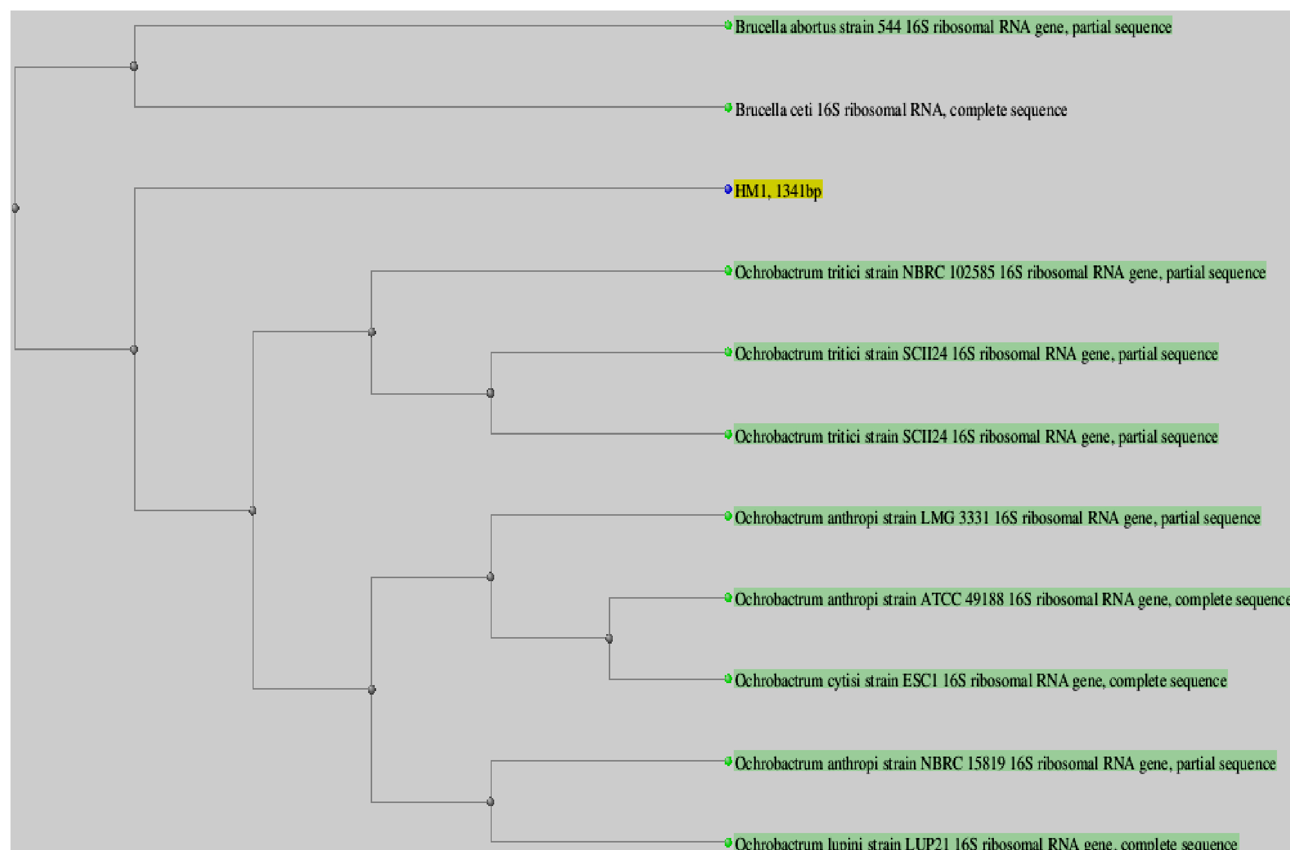


Fig. 5. Phylogenetic tree using neighbor-joining method indicating the 16S rRNA genetic link between 10 other related references microorganisms from the NCBI GenBank database and strain MH1is used as an outgroup. Species names of bacteria were followed by the accession numbers of 16S rRNA.

## CONCLUSION

Global pollution as a result of metal contamination of soil and water has severe effects on the ecosystem and human health. From the present study it can be concluded that the isolate MH1 an *ochrobactrum sp.* has a great potential to remove heavy metals as its shows to be good biosorbents and can be used for the remediation of various metals in contaminated areas therefore further characterization study of this isolate is essential.

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