Original Research Article

Screening of Heavy Metal (Lead) and Antibiotics Resistant Soil Bacteria from Myanmar Industrial Sites

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A B S T R A C T

Heavy metals are hazardous to living things and causes deterioration of the environment. Nowadays, contamination of heavy metals in the soil and water has gradually increased by human activities. This study analyzed soils from two industrial sites in Myanmar and lead tolerant soil bacteria, *E.coil* and *Alcaligenes* sp. were isolated, and characterized. MIC value of lead for *Alcaligenes* sp. was 13 mM and for *E. coil* isolates was 10 mM, respectively. Those isolates could tolerate fixed concentrations of three antibiotics (each 50 ug/ml of Chloramphenicol, Kanamycin, Tetracycline) together with 5 mM of Pb $(NO_3)_2$ and co-existence of both lead tolerance and antibiotic resistance were found. This research might somehow provide information for heavy metal and antibiotic resistant bacteria and those isolates would be good indicators for further bioremediation processes.

Introduction

Although heavy metals are natural elements present in the environment, accumulation of these elements to higher amounts are hazardous to living things and causes deterioration of the environment. Nowadays, contamination of heavy metals in the soil and water have gradually increased by human activities such as mining and smelting of metals, burning of fossil fuels, use of fertilizers and pesticides in agriculture practices, production of batteries and other metal products in industries, automobile emission, sewage sludge, and municipal waste disposal. While some heavy metals such as zinc, copper, chromium, iron, and manganese are needed in small amount for our bodies, lead, mercury, arsenic, and cadmium are the most toxic heavy metals and these poisoning can cause serious damage to our body's tissues.

Yang QQ *et al.,* (2018) surveyed soil heavy metal pollution in 402 industrial and 1041 agricultural sites throughout China and it was found that heavy metal pollution and their associated risks posed by cadmium (Cd), lead (Pb) and arsenic (As) are more serious even at very low concentrations than other heavy metals in industrial and agricultural regions in China. Pollution of soil contaminated by heavy metals can cause losses in microbial diversity and decreases the activity of

microorganisms. Soil microbial biomass was an important indicator for determination of excess soil contamination (Aceves *et al.,* 1999). Different responses of microbial population to heavy metals depend on multiple factors such as different types of heavy metals and their concentration, and tolerance on microbial species (Valls and De Lorenzo 2002). Some bacteria possess the ability to survive in the heavy metal contaminated are a sand such bacteria tolerant to specific metals might be potentially useful in bioremediation processes.

Lead is a major pollutant found in the atmosphere and is greatly toxic to human, animals, plants and environments. Lead has been widely used in batteries production, antiknock agent in gasoline, reaction tank, soldering, wire and cable insulation and jacking, radiation shields, ammunitions, stiffener in candle wicks and paint factories for many years (Zhang *et al.,* 2015). Lead pollution can cause severe effects in soil and water ecosystems such as losses in biodiversity, changes in microbial community as well as decreased pollen germination and seed viability, reduced growth and reproductive rates in plants and animals (Krishnayya and Bedi, 1986; Myra *et al.,* 2012).

In human, severe effects were caused by lead pollution to the children more than the adults and in 2016, 540,000 people were died due to lead contamination (WHO, 2016). Pollution of heavy metal becomes to threaten human life, and environment worldwide year by year. In Myanmar, as more and more industrial zones are developed nowadays and agricultural lands are contaminated with the uses of fertilizers and pesticides than before, it is needed to control pollution of heavy metals by use of remediation processes such as electrokinetic remediation, soil leaching, phytoremediation and microbial remediation.

The objective of this study is to investigate the soil analysis from two industrial sites, to identify lead tolerant bacteria and evaluate their minimum inhibitory concentrations of lead. This research may somehow benefit for further identification of heavy metals tolerant soil bacteria and microbial remediation processes.

Materials and Methods

Soil Sample collection

Soil samples were collected from two places situated near Yangon; (1) Defense Service Paint Factory (DSPF), (2) Myanmar Automobile and Diesel Engine Industries (MADI). The soil samples (0-15cm depth) were collected from each site into freshly unused polythene bags and were taken to the laboratory.

Screening, characterization and identification of lead tolerant bacteria

One ml of 10^{-5} dilution of soil suspension was plated out onto freshly prepared sterile nutrient agar medium containing 10 mg/l of Pb $(NO_3)_2$. After incubation for 24- 48 hrs at 37^0C, mucous colonies were formed over the plate and streaking plate method was used to get single colonies of pure culture. Resistant single colonies were characterized by colony morphology, staining and biochemical tests. Biochemical screening was done according to Bergey's Manual of Systematic Bacteriology (Bergey *et al.,* 1984). Biochemical tests such as IMViC (Indole, Methyl red, VP, TSI and citrate) and were performed to evaluate the enzymatic activity of isolated microorganisms.

Evaluation of lead tolerance level and antibiotics

The tolerance of bacterial isolates to lead was determined by Minimum Inhibitory

Concentration (MIC). One loop of bacterial culture was streaked on selective agar plates containing different concentrations of Pb $(NO_3)_2$ (1mM, 3mM,5mM,7mM, 10 mM, 11 mM, 12 mM, 13 mMand 14mM) of lead (II) nitrate until the isolate enabled to give colonies. The MICs value was determined after 48 hours of incubation at 37ºC (Rajbanshi, 2008).

Double screening with Pb $(NO_3)_2$ and antibiotics was performed together and fixed concentrations of each antibiotic ((50 µg/ml of Chloramphenicol, Kanamycin, Tetracycline) were used.

Results and Discussion

In this study, soil analysis test were performed by digestion method at soil analysis laboratory, Ministry of Science and Technology Research Department (MSTRD), Yangon. The result showed that soil samples from DSPF contained lead (Pb) at 0.01 % (100 ppm) and Copper (Cu) at 0.02% (200 ppm). Similarly, soil samples from MADI contained 0.01 % (100 ppm) of lead (Pb) and 0.02% (200 ppm) of Copper (Cu) but Arsenic and zinc were not detectable in both industrial sites (Table 1). Lead is considered as hazardous element and maximal permissible addition (MPA) value for lead is 55 ppm (55 mg/kg) (Vodyanitskii *et al.,* 2016). Therefore, it was concluded that industrial sites in Myanmar were starting to be contaminated with lead and it is needed to regard as a major concerning issue for controlling contamination of heavy metals. Further examinations are needed to carry out in other industrial zones which might also be contaminated with heavy metals and the cleanup strategy are necessary to apply for eliminating the pollution.

This soil analysis results allowed to be focused on bacteria tolerant to lead and copper and the present study only carried out to isolate and characterize lead tolerant bacteria. According to morphological and microscopic morphology (Table 2 and Table 3), rod-shaped gram-negative bacteria were found in two collected sample sites. When basic biochemical characteristics such as MR, VP, Citrate, Indole, TSI tests were used to identify the isolates, we can characterize that the bacteria found in these collected soil samples were *E. coil* and *Alcaligene* sp (Table 3 and Figure 1-2). Several previous studies also indicated that lead resistance in many indigenous microorganisms including *Bacillus* sp., *Pseudomonas* sp., *Acinetobacter* sp., *Flavobacterium* sp., *Escherichia coli* and *Alcaligenes* sp. have been identified from the lead contaminated soil (Trajanovska S *et al.,* 1997; Tamil Selviet *et al.,* 2012).

For evaluation of tolerant level to lead (II) nitrate, it was found that Minimum Inhibitory Concentrations (MIC) of *Alcaligenes* isolate was 13mMand that of *E.coli* isolate was 10 mM revealing these isolates were designated to be used as good candidates for further bioremediation research. Similarly, the MIC of lead was found to be 60-80 ppm for *E.coil* and and MIC for *Alcaligenes* sp. was 80 ppm (Tamil Selvit *et al.,* 2012) and it seemed *Alcaligene s*sp might have possibility to tolerate heavy metals than *E.coli*. Irawati *et al.,* (2017) identified indigenous heavy metal tolerant bacteria from the industrial sewage and the highest lead tolerant bacteria showed MIC value of 11 mM showing those isolates were used for further study of lead contamination control. Microorganisms can interact with heavy metal via many mechanisms and different level of lead tolerant level might be due to different types of mechanisms such as bioaccumulation and biosorption processes within each isolates (Table 4).

In recent studies, co-existence of both heavy metal tolerance and antibiotic resistance was observed in a number of bacteria and that two

/more co-resistance genes could be frequently found on mobile elements such as plasmids and transposons as well as also on chromosome. Therefore, double screening method for checking linkage of these heavy metal and antibiotic resistant genes were tested by using fixed concentrations of three antibiotics (Chloramphenicol, Tetracycline, and Kanamycin) together with 5mM, 7mM and 10mM lead (II) nitrate in selective media. It was observed that both *Alcaligenes* sp and *E.coil* isolates showed resistance to each 50 ug/ml of three kinds of antibiotics but MIC level of lead tolerance was reduced to 5 mM lead (II) nitrate. It was possible that these wild type isolates were not adapted to more than 5 mM lead (II) nitrate compound at fixed concentrations of three antibiotics. Although the cell was struggling against two stresses at the same time, by carrying both genes for heavy metal (lead) and antibiotic resistances, it was regarded that these isolates contained both heavy metal and drug resistant genes and somehow, soil from two industrial sites were contaminated with both heavy metals and antibiotics. In many natural environments, the emergence of metal resistance and multidrug resistance in bacteria has been observed due to co-contaminations of heavy metal and antibiotics (Chen *et al.,* 2015) and it might be a major issue for controlling pollution and infectious diseases (Table 5).

Table.1 Soil sample analysis of heavy metals by digestion method

Places	Lead	Copper	Arsenic	Zinc
DSPF	0.01%(100ppm)	0.02%(200ppm)	ND	ND
MADI	0.01%(100ppm)	0.02%(200ppm)	ND	ND

DSPF=Defense Service Paint Factory
MAID=Myanmar automobile and Diesel Engine Industries
ND=Non-detected

Table.2 Study of colony morphology and microscopic morphology

Morphological characters	*E.coli* isolates	*Alcaligenes* sp isolates
Colony Morphology		
Color	-Pink colony on MacConkey agar	-No pigment
Shape	-Circular	-Circular
Surface	-Smooth	-Smooth
Elevation	-Flat	-Flat
Edge	-Entire	-Entire
Opacity	-Translucent	-Opaque
Gram-stain	-Negative	-Negative
Microscopic Morphology		
-Cell size	-0.6 µm-2-6 µm	-0.5-1.2µm-0.5-2.6µm
-Shape	-Single rods, coccal rods	-Rods, single, in pairs or in short chains
-Motility	- +	- +

Table.3 Biochemical tests of two isolates

Biochemical characteristics	*E. coli* isolates	*Alcaligenes* isolates
TSI	-	+
Indole	-	+
MR	-	+
VP	-	-
Citrate	+	-
Catalase	+	+
Growth on Mac Conkey agar	+	+
Growth at 42^0C	-	+
Aerobic	+	+
Gelatin liquifaction	+	+

Table.4 Evaluation of Minimum Inhibitory Concentration (MIC) to Lead (II) Nitrate without antibiotics

Concentrations of $Pb(NO_3)_2$	*E. coli* isolates	*Alcaligenes* isolates
1mM	+	+
3mM	+	+
5mM	+	+
7mM	+	+
10mM	+	+
11mM	+	+
12mM	-	+
13mM	-	+
14mM	-	-

Table.5 Double Screening of two isolates with Pb $(NO_3)_2$ and three antibiotics

Concentrations of $Pb(NO_3)_2$	Concentrations of three antibiotics	*E.coli* isolates	*Alcaligenes* isolates
5mM	50ug/ml each	+	+
7mM	50ug/ml each	-	-
10mM	50ug/ml each	-	-

Three antibiotics (Chloramphenicol, Kanamycin, Tetracycline)

Fig.1 (a) IMViC test of *Alcaligenes* sp isolate (b) IMViC test of *E.coli* isolate
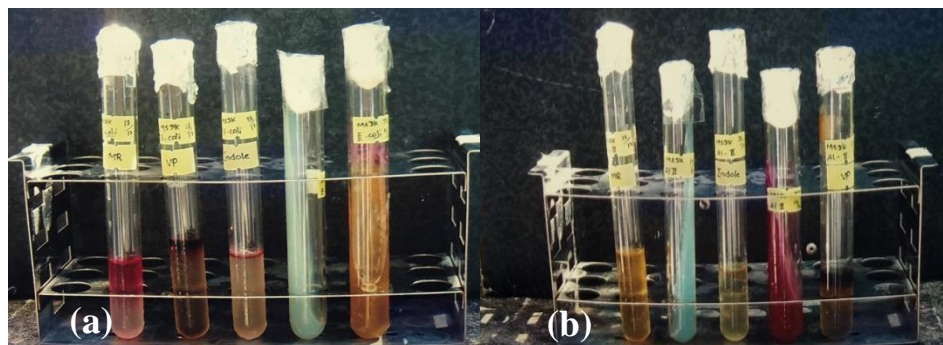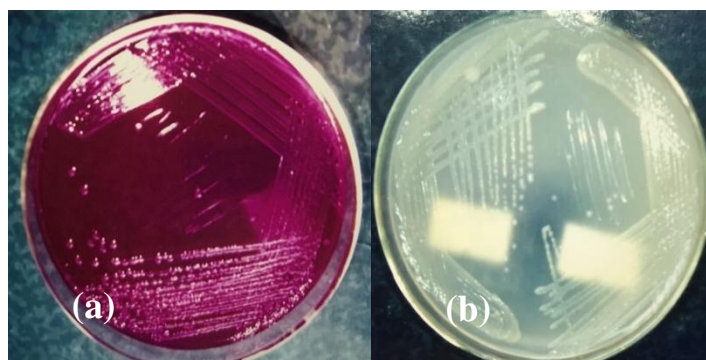

Fig.2 (a) Pure culture of *E.coli* on Mac Conkey agar medium (b) Pure culture of *Alcaligenes* sp. on selective agar medium

As it was obviously found the co-existence of heavy metal resistant genes and antibiotic resistant genes together in the collected isolates, these two genes might be located either on the same chromosome or on the same plasmid. When further study was done by extracting the plasmid DNA from these double screening isolates, it was observed that colonies of wild types *E. coli* isolate possessed approximately 2.3 kb of plasmids in size whereas wild type *Alcaligenes* sp. had large plasmids about 23 kb in size (Data not shown). Therefore, it can conclude that heavy metal resistant genes and antibiotic resistant genes were together on mobile genetic elements, plasmids and plasmids aid the bacteria for tolerance and resistance mechanisms against heavy metals and antibiotics in the pollutant environment.

In conclusion, this study indicated that bacteria with lead tolerant and three kinds of antibiotic resistance were observed in soils from two industrial sites. Contaminated sites are sources for microorganisms with heavy metal tolerant activities and the bacterium which is multiple tolerant to many heavy metals can be effectively used as a biosorbent in the pollutant area with more than one type of heavy metals (Irawati *et al.,* 2017). Therefore, these isolates would be good indicators for bioremediation processes and further analysis of those lead tolerant isolates with other hazardous heavy metals should be also examined.

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