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Isolation, identification and characterization of potential bacteria from Malaysian wastewater showing resistance to cadmium, copper and iron

Tendulkar A [1,]*, Yan Yi Wei [2], Murali Krishnan V [3]

[1]*Ph.D Candidate, Biosciences Department, Malaysia University of Science and Technology, Petaling Jaya, Selangor, Malaysia-47301*
[2]*Lecturer, Biosciences Department, Malaysia University of Science and Technology, Petaling Jaya, Selangor, Malaysia- 47301*
[3]*Assistant professor & Head, Dept. of Biochemistry, Government Arts College, Paramakudi, Tamilnadu, India-623701*

Abstract: Indigenous species of microorganism provides a potential system for the treatment of metal contaminated water. The current research work was carried out to study the wastewater collected from treatment plant for its physio-chemical and isolation and characterization of microbes showing resistance to selected heavy metals. A total of 25 isolates were screened, using LB agar and the metal resistant organisms were selected using differential agar media containing selected concentration of cadmium, copper and iron, which showed optimum growth at 30°C after 24- 48 hours of incubation. The isolated microbial cultures were further tested against varying concentrations of heavy metals (0.019 – 40 mM) using serial dilution technique. The selected organisms that showed higher resistance to heavy metals were further tested using 16Sr RNA sequence. By using bioinformatics tool MEGA6 and BLAST the isolates were identified to be closely related to the members of genus as Actinobacter, Arthobacter, Bacillus and Pseudomonas. This result thus reveals that the identified microorganisms have genes of resistance towards heavy metal. It is therefore, recommended that the identified bacterial strains are the best choice to ensure heavy metal remediation under metal stresses especially when domestic wastewater effluents are involved.

Key words: Minimal inhibitor concentration; 16Sr RNA gene sequence; MEGA6; BLAST; Phylogenetic tree

1. Introduction

The presence of heavy metals in sewage and wastewater are considered as a major hazard to natural water, animals and human health. Certain metals such as copper (Cu) and iron (Fe) are known to be essential nutrients sources, but higher concentrations of these metals have deleterious effect on the environment (Rajbanshi, 2008). Cadmium (Cd) is a non-essential metal and poisonous for plants, humans and animals (Raja et al., 2009). The most severe disease caused by cadmium poisoning is 'Itai- Itai' in Japan. Chronic poisoning causes proteinuria and formation of kidney stones (Borges et al., 2003). The common sources of heavy metal contamination are from effluents of metal based industries, chemical industries, sewage and various processes as well as operations like electroplating, milling, cutting, rubbing, polishing etc.

To remove metal pollution from the environment, various remediation methods wherein bacteria, fungus and plants are used (Singh et al., 2014), but bacteria are extensively used for this (Pushkar et al., 2015). The selection of microbes depends on numerous factors; one of the major factors is the pollutant to which the microbe will be remediating (Singh et al., 2014). Since there is various type of heavy metals available in the earth atmosphere, it is not possible for one single species of bacteria to tackle all, but depending on the heavy metal pollution, there are various species of bacteria available to remove excess heavy metal from the environment (Sharma et al., 2009; Mohideen et al., 2010). Many research studies are focused on the removal of heavy metals from the environment due to its toxicological threat than other pollutants (Fenglian et al., 2011; Sheela et al., 2014). In the current research work, the focus will be on the identification, isolation and characterization of heavy metal (cadmium, copper and iron) tolerant bacterial species from Malaysian wastewater.

2. Methodology

2.1. Sampling, physio-chemical analysis and microbial isolation

Wastewater samples were collected from different location in Damansara and Pantai, Selangor, Malaysia. The collected samples were stored in sterilized plastic containers at 4°C, as per the standard procedure for water collection (Pani, 2007). The sample were further analyzed for colour, odor, pH, electric conductivity (E.C), dissolved

* Corresponding Author.

oxygen demand (D.O), biochemical oxygen demand (B.O.D) and chemical oxygen demand (C.O.D).

To isolate bacteria resistant to metal ions (cadmium, copper and iron), the collected samples were spread plated on a Luria Bertani (LB) agar plate supplemented with 5 ppm of cadmium, copper and iron (Raja et al., 2009). Luria Bertani (LB) agar and Potato Dextrose agar plates were used as positive control (Raja et al., 2009). Plates were incubated at 30°C for a week and colonies showing different morphological characteristics are selected and used for further analysis, such as MIC and for identification.

2.2. Heavy metal sensitivity of the isolated bacteria

Heavy metal sensitivity test for cadmium, copper and iron was performed according to the method mentioned by Ranjitha et al. (2011). In the method, 96 well microtitre plate is used wherein, 100 µl of metal ions was added to wells 1-7 of row A, at a concentration of 40 mM and 50 µl of sterile normal saline was added to rows B- L. Followed by transferring 50 µl from row A to the wells of row B, repeated until twelve serial dilutions (until row L). Further 40 µl of broth and 10 µl of bacterial suspension were added into the wells. The plate was covered and incubated at 30°C for 24 hours.

2.3. Isolation of genomic DNA and identification of the bacterial isolate

Genomic DNA of the isolates was extracted using Qiagen genomic DNA extraction kit. By using a pre-determined universal primer 16S rRNA; forward 27f (5′-GAGTTTGATCACTGGCTCAG-3′) and reverse 1492r (5′-TACGGCTACCTTGTTACGACTT-3′) (He et al. 2010; Altimira et al., 2012) was used for analysis of 16S rRNA genes. PCR was performed using 50 µl of reaction mixture from Bioline *Taq* polymerase master mix kit. The reaction was carried out in 50 µl reaction volume containing 1 µl of DNA template, 0.5 µl of each primer, 23.2 µl ultrapure water and 25 µl of Taq master mix (MyTaq Master Mix PCR Kit, Bioline Inc, USA). PCR reaction was carried out using PCR thermal cycler (MJ Research), with following programme; initial denaturation at 94°C for 3 minutes, denaturation at 95 °C for 1 minute, annealing at 55 °C for 30 seconds, extension at 72 °C for 1 minute and final extension at 72 °C for 5 minutes for 30 cycles. The amplicon was then run on 1.2% Agarose using gel electrophoresis unit (Thermofisher, USA). A band size of 1500 bp was obtained, which was extracted using gel extraction kit (Qiagen, USA) before sending it for sequencing. The sequenced product was analyzed using MEGA5 software for alignment and BLAST tool.

3. Result and discussion

The wastewater samples immediately after collection were subjected to physiochemical and microbial analysis. The result of the physiochemical tests (table 1) showed the sample had higher electric conductivity and lower oxygen level. The less amount of oxygen in any water body is an indication of pollution due to human and animal activities (Patil et al., 2012). A higher electric conductivity indicates presence of higher amount of salts which could be due the presence of animal wastes (Ekanem et al., 2016). The pH determines the acidic and alkaline content of a water sample, thus a pH ranging in near neutral usually support good growth of microbes. Microbial analysis was done using L.B agar supplemented with 5 ppm of cadmium, copper and iron. Total of 25 colonies were isolated from the wastewater.

Table 1: Physiochemical characterization of metal contaminated samples

Parameters	DOE Standard	Site 1	Site 2	Site 3	Site 4
Color	Colorless	Black	Black	Black	Black
Odor	Odorless	Pungent	Pungent	Pungent	Pungent
Temperature (°C)	25	21.6	22.3	21.1	22.5
pH	7 (neutral)	6.76	5.99	6.86	6.67
E.C (µS/cm)	10.00	13.13	13.41	13.2	13.39
D.O (mg/ml)	7.00	5.35	5.97	5.55	4.08
B.O.D (mg/ml)	91- 100	0.80	2.09	0.80	0.60
C.O.D (mg/ml)	10000	4967	4883.50	4750	4750

SD: ±0.01

3.1. Screening of cadmium, copper and iron resistant strain

Bacteria isolated from the wastewater showed high resistance towards all three metal ions (Cd, Cu and Fe). Among the 25 isolates only 4 of the isolates (labeled PW6, PS3, DS4 and DS8) were sensitive to iron, whilst one (DW4) toward copper (Table 2). Thus among the three heavy metal ions, the isolates showed maximum resistance towards cadmium and copper.

Total 20 bacteria out of the 25 isolates showed tolerance towards the entire three metal ion (Cd, Cu and Fe), which were further analyzed using 16Sr RNA analysis for the identification. Resistance of the 20 isolates towards heavy metal ions was in the following order: Cadmium> Iron> Copper.

Table 2: Minimal inhibitor concentration of heavy metal for bacteria isolated from wastewaters

Strain ID		Heavy metal ion (Concentration in mM)		
		Cadmium	Copper	Iron
1	PW1	40.00	5.00	40.00
2	PW2	40.00	40.00	40.00
3	PW3	40.00	0.62	40.00
4	PW4	40.00	40.00	40.00
5	PW5	40.00	40.00	40.00
6	PW6	40.00	0.62	-
7	PW7	40.00	40.00	20.00
8	DW1	5.00	40.00	40.00
9	DW2	40.00	40.00	40.00
10	DW3	40.00	40.00	40.00
11	DW4	10.00	40.00	-
12	DW5	40.00	40.00	20.00
13	PS1	10.00	40.00	5.00
14	PS2	20.00	40.00	40.00
15	PS3	40.00	40.00	-
16	PS4	10.00	40.00	40.00
17	DS1	40.00	40.00	40.00
18	DS2	40.00	40.00	40.00
19	DS3	40.00	40.00	40.00
20	DS4	40.00	-	40.00
21	DS5	20.00	40.00	40.00
22	DS6	20.00	40.00	40.00
23	DS7	40.00	2.50	20.00
24	DS8	40.00	40.00	-
25	DS9	40.00	10.00	40.00

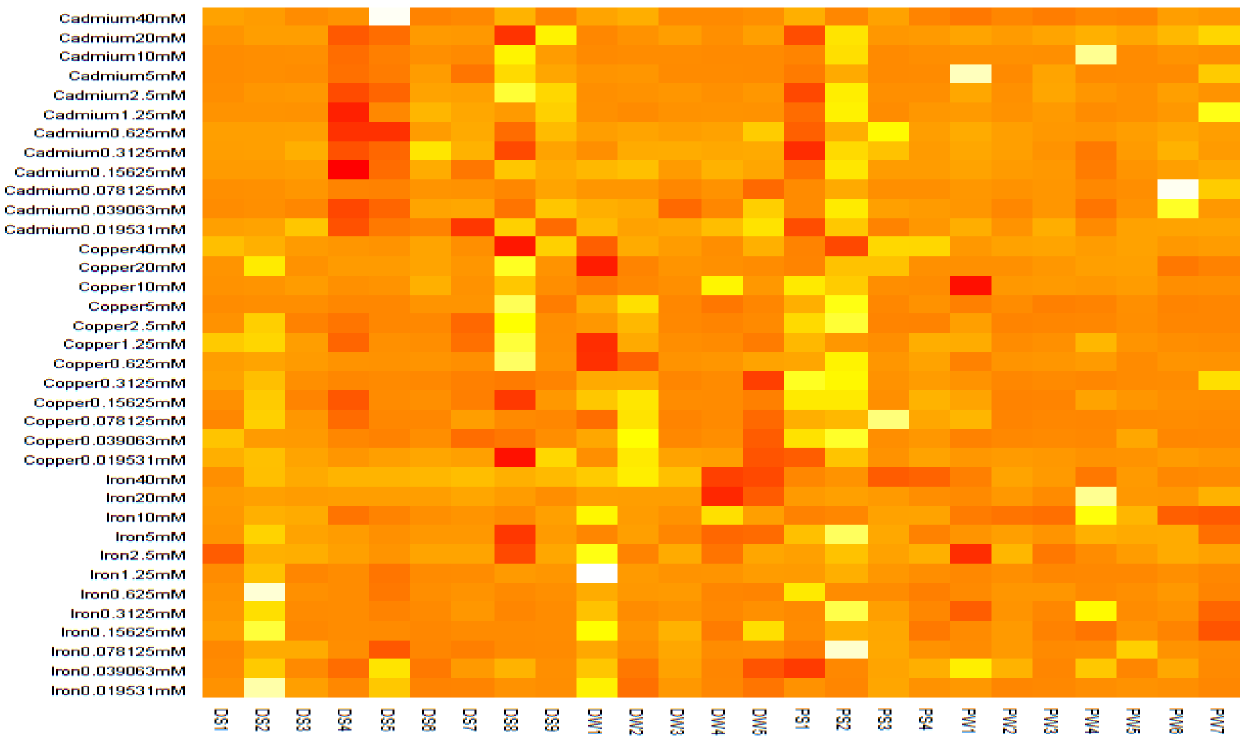

Fig 1: A heat map of the 25 isolated is plotted showing metal (cadmium, copper and iron) reaction in a concentration of 0.01 to 40 mM on the growth of the bacteria.

A heat map is plotted (Fig. 1) after normalizing the data of the 25 isolates against the heavy metal ions of cadmium, copper ad iron in a concentration range from 0.01 to 40 mM. This heat map signifies the growth pattern of the bacteria in tolerating heavy metal ions at different concentrations. The same result was observed by Nath et al. (2012), wherein the different species of bacteria showed high tolerance towards various heavy metal ions. This result helps in indicating the probability of the presence of metal resistance gene in the bacteria, due to which the bacteria can tolerate an environment with higher concentration of heavy metals (de Silva et al., 2012)

3.2. Analysis of sequencing results

After performing the sensitivity test to screen out only the strains showing resistance to Cd, Cu and Fe, it was observed that only 20 such bacterial strains out of 25 have showed heavy metal resistance. These 20 strains were tested further for identification. By using 16SrRNA analysis the sequence was comparatively analyzed with already available database (NCBI), which showed that the strains were closed to *Acinetobacter sps, Arthobacter sps, Bacillus amyloliquefaciens, B. aryabhattai, B. cereus, B. subtilis, B. sps CZ22, B. thuringiensis, Pseudomonas sps PP103, P. lundensis, P. psychrophila, P. miqulae and P. panacis* (Fig. 2).

When analyzing the sequence, a novel species *Bacillus aryabhattai* was found among the isolates. Shivaji et al. (2009) first isolated it from the earth's upper atmosphere in a cryotube. Later, it was also found in the deep-sea of the South China Sea, which can produce glucose and fructose by degrading bagasse (Shivaji et al., 2009). The research paper by Chen et al. (2012) was on the distribution of culturable endophytic bacteria in aquatic plants and their potential for bioremediation in polluted water, wherein the author was able to isolated *Bacillus aryabhattai* from the phytoremediating aquatic plants. It has been reported that endophytic bacteria such as *Bacillus aryabhattai* can enhance the phytoremediation of xenobiotic compounds in polluted environments and increases the solubility of phosphorus, thus promote the growth of the host plants (Ryan et al., 2008).

Apart from *Bacillus aryabhattai*, other species of *Bacillus*, *Acinetobacter, Arthobacter* and *Pseudomonas* are also well studied and found to be tolerant towards heavy metal ion (Sharma et al. 2009; Raja et al., 2009; Mohideen et al., 2010). After analyzing the MIC result and 16Sr RNA analysis 15 potential bacteria species compared to the 20 identified species were found to be showing good resistance towards heavy metal. These bacteria were closely related to *Arthobacter sps, Bacillus aryabhattai*, *B amyloliquefaciens, B. cereus, B. circulan, B. subtilis, B. thuringiensis, Pseudomonas fargi, P. lundensis, P. miqulae, P. panacis, P. psychrophila, P. rhodesiae, P. sps.*

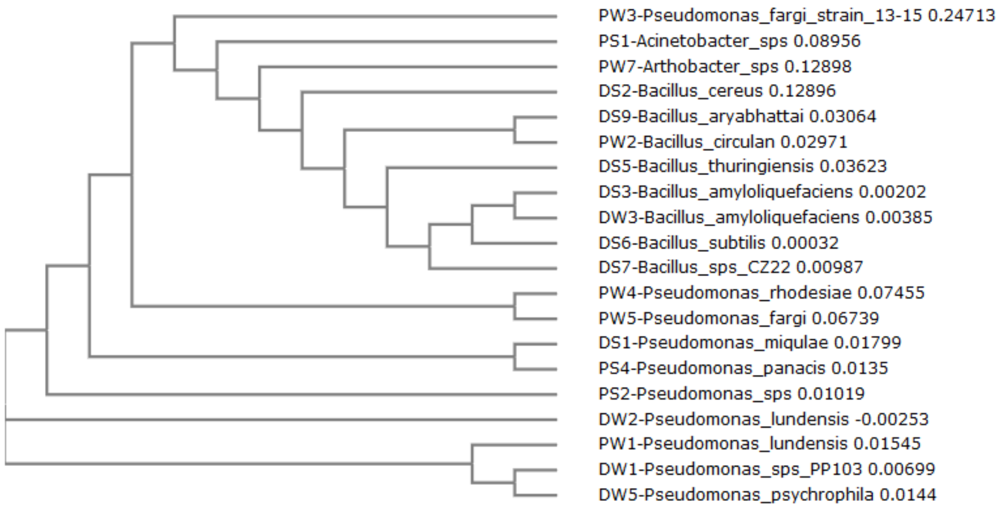

Fig. 2: The figure illustrates a phylogenetic tree diagram of the 20 identified sequences by comparing the sequence with the already available sequence in NCBI (online database).

4. Conclusion

The use of bacteria as a tool for bioremediation is a very useful, convenient and cost-effective method. There have always been needs for the identification and isolation of new species of bacteria, which are showing resistance to pollutants such as heavy metals, ammonia, hydrocarbons etc.

In the current study, 25 isolates from wastewater were characterized to test their resistance against cadmium, copper and iron. Out of which 20 isolates showed a promising result, which were further identified. The MIC results and the PCR based bacterial identification clearly gives an idea that these species of bacteria have genes conferring resistance to Cd, Cu and Fe, thus can be considered for the bioremediation of these heavy metals in the environment.

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