Pertanika J. Trop. Agric. Sci. 41 (2): 699 – 714 (2018)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Use of Bio-Chemical Surfactant Producing Endophytic Bacteria Isolated from Rice Root for Heavy Metal Bioremediation

Arun Karnwal

Department of Microbiology, School of Bioengineering and Biosciences, Lovely Professional University, Punjab 144411,India

ABSTRACT

A variety of microorganisms generate highly potent surface-active bio-molecules or biosurfactants, which vary in their chemical properties and molecular size. In the present study, bioremediation effect of *Pseudomonas fluorescence* RE1 (GenBank: MF102882.1) and RE17 (GenBank: MF103672.1) endophytes on heavy metals Zn, Cr, Cd, and Ni were investigated. A total of 56 morphologically distinct isolates from indigenous rice roots were selected and subsequently characterised genotypically by using 16S rRNA sequencing approach. Next, biosurfactant production and heavy metal removal ability by the isolates were screened on the basis of α and β hemolysis on blood agar plates, BATH assay, and CTAB method. Analysis of bioremediation of heavy metals was done by using atomic adsorption spectroscopy. Bioremediation analysis revealed that isolates RE1 and RE17 reduced the concentration of Zn by up to 92% and 90% at pH 7.5, respectively, while for Ni, % removal was the same for both strains at 95% at pH 7.5. Biosorption results for Cr and Cd showed highest metal removal efficiency by *Pseudomonas fluorescence* RE17 at pH 8, 92% and 98%, respectively. Both isolates showed significant metal removal efficiency at 32±1 °C for all experimental heavy metals. The present study suggests that all endophytes withstand at high concentration of testing heavy metals and can be used for bioremediation of heavy metals in contaminated environments.

Keywords: Biosurfactant, endophyte, plant growth promoting, phytohormones, rhamnolipids

ARTICLE INFO

Article history:
Received: 20 June 2017
Accepted: 30 November 2017

E-mail address:
arunkarnwal@gmail.com (Arun Karnwal)

ISSN: 1511-3701

INTRODUCTION

In past years, rapid industrialisation and its dependency on chemicals have generated and added various types of pollutions in the environment. This environmental pollution is becoming a very serious issue

of public health and ecology of many biotic factors involved in maintaining and balancing the earth's ecosystem (Song et al., 2016). Heavy metal pollution is one of the examples of industrial pollution caused by various industrial and military activities (Amer et al., 2015; Robles-Gonzalez et al., 2008) during electroplating, metalworking, refinishing, drilling, and explosives production. Availability of heavy metals (Cd, Cr, Cu, Ni, As, Pb, and Zn) in soil or water may cause various hazardous effects in living organisms that cause multiple level of complications such as paralysis, seizures, brain damage, nerve damage, pericapillary hemorrhages, mesh lines on finger nails, adult respiratory distress syndrome, gastrointestinal upset, hemolysis, anemia, hypotension and other life-threatening complications (Chen et al., 2017; Salmani & Fazaelipoor, 2016). Therefore, removal of heavy metals from the environment is necessary for the safety of all biological entities.

In recent years, the use of biologically derived surfactants has attracted more attention for heavy metal removal from the environment over chemical surfactants. The reason for their preference over chemical surfactants are due to their less toxicity, biodegradability, environmental friendly, better foaming ability, more selectivity, effectively able to act at various temperature, pH and salinity conditions (Juwarkar et al., 2007; Kassab & Roane, 2006; Kim et al., 2008). Biosurfactants are surface-active chemicals produced by either bacteria or fungi. They tend to accumulate at the interface of immiscible fluids and surfaces, where they reduce surface tension as well as interfacial tension (Parkinson, 1985). Additionally, it plays a significant role to make the environment clean from pollutants and contaminations (Zouari et al., 2017). These bio molecules can be formulated from various waste materials, therefore, their production is more economical and feasible in a large setup (Karnwal, 2017). Biosurfactants have a broad range of applications in industries and homes such as detergent, emulsification, frothing, moistening, penetrating, metal segregation, resource recovering and food additives (Lizardi-Jimenez & Hernandez-Martinez, 2017). Biosurfactants are produced by a wide range of microorganisms and therefore differ in their chemical structure and activity. Endophytes are defined as microorganisms that are found in the living tissues of plant hosts and do not cause harmful effects on the plants (Han et al., 2011). Biosurfactants are among the possible biological molecules isolated from these microorganisms.

Therefore, the aim of the present study was to isolate heavy metal resistant endophytes from rice roots, analyse the PGP traits of isolates and to evaluate their bioremediation effectiveness under *in vitro* conditions.

MATERIALS AND METHODS

Isolation of Heavy Metal-Resistant Endophytic Bacteria from Rice Root

A total of 40 healthy rice (*Oryza sativa* L. Basmati) plants were randomly selected and collected from agriculture field situated at Dehradun (30° 19' N, 78° 04' E) Uttarakhand, India. The collected plants were thoroughly washed to remove dust and other physical contaminants, and subsequently rinsed with sterilised distilled water (Karnwal, 2009). Sterile scalpel was used to dissect the root and shoot of the rice plants. Surface sterilisation of dissected roots was achieved by washing them with 95% ethyl alcohol followed by 3% sodium hypochlorite for two to three minutes (Govarthanan et al., 2016; Liu et al., 2014). The roots were washed again with sterile distilled water several times to remove sodium hypochlorite from the surface. After thorough rinsing, the surface-sterilised roots were dissected into small pieces Then, 1 gram of fresh weight root tissue was ground in sterile mortar and pestle with 0.85% sterilised saline solution. The ground tissue extract was serially diluted (sevenfold) in sterile saline and 100 µl aliquots were spread on nutrient agar plates (Hi-Media, India). These plates were incubated at 28±1°C for 48 hours. After incubation, morphologically distinct bacterial colonies were separately streaked on nutrient agar plates for the isolation of pure culture of endophytic bacteria for biosurfactant production and heavy metal study.

Heavy Metal Tolerance

Mineral salt medium (MSM) supplemented with 1ml of heavy metal (Zn, Cr, Cd and Ni) solution was used for screening the heavy metal tolerance ability of isolated endophytes. Various concentrations (ranging from 50 to 800 mg l^{-1}) of heavy metals ($ZnSO_4.7H_2O$, $K_2Cr_2O_7$, $3CdSO_4.8H_2O$ and $NiSO_4.6H_2O$) were used. Isolated endophytes were streaked and incubated on MSM plates supplemented with heavy metal (sterilised by filtration) at 32±1°C for 48 hours (Kamala-Kannan & Krishnamoorthy, 2006). The lowest concentration of the metal that completely inhibited the growth of bacteria was considered as minimal inhibitory concentration (MIC). A total of 25 bacterial isolates were selected for further study based on heavy metal tolerance study data.

Haemolysis Assay

All the 25 endophytes were purified and selected for biosurfactant production on blood agar plates. A pure culture of isolates was streaked on blood agar plates and incubated at 37±1°C for 24 hours. Bacterial colonies with clear zone of haemolysis were selected and reported as positive for biosurfactant production ability (Carrillo, Mardaraz, Pitta-Alvarez, & Giulietti, 1996). These colonies were further analysed to screen the ability of biosurfactant production and heavy metal removal optimisation at pH and temperature.

Bacterial Adhesion to Hydrocarbons (BATH) Assay

Microbial adhesion to hydrocarbons was performed as described by Rosenberg, Gutnick and Rosenberg (1980) with some modification. Individual endophytic bacterial suspension (2 ml) was dispensed in 20 ml volume screw capped culture tubes with a varied amount of hydrocarbon (hexane and crude oil) ranging from 0.1, 0.2, 0.3, 0.4 and 0.5 ml. Cell suspension and hydrocarbon mixture were vortexed for three minutes and allowed to equilibrate for 60 minutes for the completion of the biphasic formation. Cell adherence percentage to hydrocarbon was determined at 600 nm using a spectrophotometer as described by Van der Vegt, Van der Mei, & Noordmans (1991):

$$1 - (\text{OD of the aqueous phase/OD of initial cell suspension}) \times 100$$

CTAB Assay

Siegmund and Wagner (1991) described a new method for the detection of extracellular glycolipids or other anionic surfactants on agar plates supplemented with cetyl-tri-methyl ammonium bromide (CTAB). For the detection of extracellular glycolipids, blue agar plates enriched with 200 µg ml^{-1} cetyl-tri-methyl ammonium bromide (CTAB) and 5 mg ml^{-1} methylene blue were streaked with the endophytic bacteria isolates (Siegmund & Wagner, 1991). The plates were then incubated at 37±1°C for 24 hours. Colonies with blue halos were marked as positive for biosurfactant and were selected for further study. On the basis of the hemolytic, BATH and CTAB assay, three out of the 25 bacterial isolates were selected as highly potential biosurfactant producers for further studies. These colonies were named as RE1, RE6, and RE17.

Biosurfactant Production

Biosurfactant production was carried out as described by Karnwal (2017). For biosurfactant production, sugar cane waste supplemented with 10% glycerol was used as nutrient media. 10 ml of inoculum of each isolate (RE1, RE6, and RE17) was added into 100 ml of fermentation medium in Erlenmeyer flasks (in triplets). These flasks were incubated at 37±1°C for 24 hours at 150 rpm. After the incubation period, the fermentation broth was centrifuged at 5000 rpm for 10 minutes to obtain cell-free culture. The effectiveness of the biosurfactant was determined by Drop collapsing test, Oil-spreading test, and Emulsification index assay as described by Sarubbo, Luna and de Campos-Takaki (2006), and Morikawa et al. (1993).

Drop Collapsing Test

A qualitative approach was used to detect the biosurfactant production by the endophytes isolates as described by Jain, Collins-Thompson, Lee, & Trevors (1991). The drop collapsing assay depends on the destabilisation of liquid droplets by biosurfactant. Crude oil was used for this assay, where 96 well microtitre plate lid was filled with 2 µl of crude oil and equilibrated

for 24 hours at room temperature. Then, 4 µl of the cell-free culture was added to the surface of oil and drop size was observed after 1 minute. The result was considered positive when the drop was flat and negative for rounded drops (Youssef et al., 2004).

Oil-Spreading Assay

To examine the effectiveness of biosurfactant (cell-free culture), the lower lid of a petri plate was filled with 15 ml of sterile distilled water and 15 µl of crude oil was gently spread on the surface of the water layer. One drop of the extract was added to the surface of oil layer as described by Morikawa, Hirata, & Imanaka (2000). The size of oil free zone was recorded for each isolate to assess biosurfactant activity (Morikawa et al., 1993).

Emulsification Index (E24) Assay

The emulsifying capacity was evaluated by an emulsification index (E24) assay. E24 was determined by adding 2 ml of hexane or xylene in 2 ml of cell-free extract for each isolate in a separate test tube. These tubes were vortexed for two minutes and allowed to stand for 24 hours. Emulsification index percentage was then calculated as described by Sarubbo, et al. (2006) using the following equation:

$$E24 = \text{height of emulsion formed} \times 100/\text{total height of solution}$$

Effects of Temperature and pH on Biosorption of Heavy Metals

Effects of temperature and pH on heavy metal biosorption were determined with RE1 and RE17. 100 ml of MSM supplemented with individual heavy metal (50 mg l^{-1} concentration) was separately inoculated with 10 ml of bacterial isolates RE1 and RE17 at several pH levels, ranging from 5 to 9 in Erlenmeyer flasks. These flasks were placed and incubated in a shaking incubator (200 rpm) at 32±1°C for 24 hours. After incubation, samples were collected from each flask and centrifuged at 10000 rpm for five minutes. Cell-free extract was filtered with Whatman No. 1 filter paper and analysed for residual heavy metal concentration by using atomic adsorption spectroscopy. Similarly, individual flask with a 100 ml heavy metal solution and 10 ml bacterial isolate was placed at different temperature (24, 28, 32, 36 and 40°C) for 24 hours and remaining heavy metal concentration in cell-free broth was analysed by atomic adsorption spectroscopy. All experiments were conducted in triplicates and the average mean was calculated from recorded data.

16s rRNA Sequencing

16S rRNA sequencing and phylogenetic analysis were done for both isolates RE1 and RE17. Universal 16S rRNA primers (8F 5’ AGAGTTTGATCCTGGCTCAG 3’ and U1517R 5’ ACGG(A/C) TACCTTGTTACGACTT 3’) were used for 16S rRNA amplification of bacterial isolates under PCR reaction (Srinivasan,

Karaoz, Volegova, MacKichan, & Kato-Maeda, 2015). Primers were checked for specificity using the probeBase online utility and the BLAST search facility at the NCBI (Genbank database).

RESULTS AND DISCUSSION

Isolation of Heavy Metal-Resistant Endophytic Bacteria from Rice Root

Endophytic microbes are associated with the interior of vegetal tissues of plants without causing any damage or producing external structures (Ji et al., 2010). In the present study, a number of endophytes were isolated from rice. After complete surface sterilisation by ethanol and sodium hypochloride, crushed root tissues of rice were serially diluted and plated on nutrient agar plates for the isolation of endophytes. After incubation 2.70 X 10^2 CFU ml^{-1} bacterial cells were recovered from NAM plates and 5.6 x 10^1 CFU ml^{-1} morphologically distinct bacteria were purified and selected for further investigation for heavy metal tolerance and biosurfactant production ability.

Heavy Metal Tolerance

Heavy metals do not have any vital role for living organisms and are highly poisonous even at an immensely low concentration (Khan & Bano, 2016). In contrast to essential metals, heavy metals negatively affect the physicochemical and biochemical activities of microbial population and ultimately lead to reduced biomass and diversity of microorganism in soil (Kumar et al., 2015). In the present study, 56 endophytic bacteria were streaked on several concentrations of heavy metal on MSM plates. Results indicated that 40 isolates from 56 bacterial strain showed positive results for heavy metal tolerance. A total of 25 isolates showed high MIC for all heavy metals tested (Figure 1) and selected for biosurfactant and heavy metal removal analysis. All the 25 isolates were named with RE1 to RE25 for record maintenance and documentation.

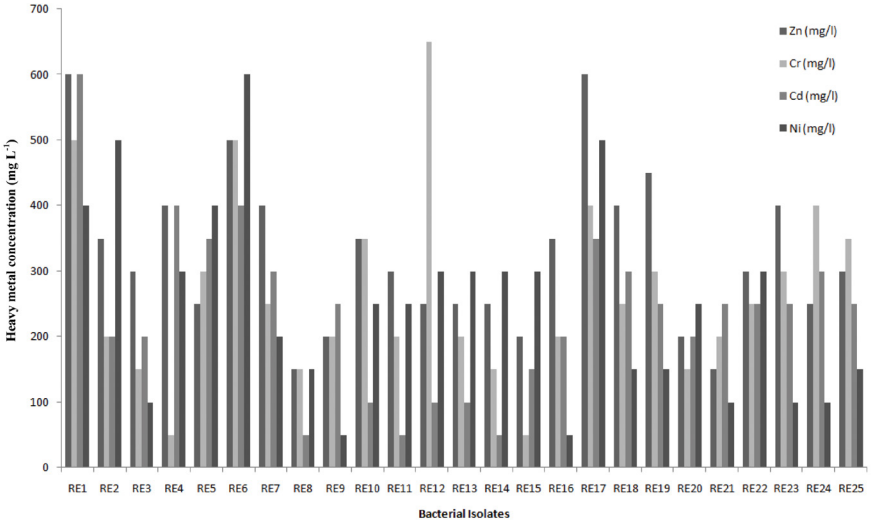


Figure 1. Heavy metal tolerance result of endophytic isolates

Haemolysis Assay

All the 25 bacterial isolates were streaked on blood agar plates to observe erythrocytelysis. According to Carrillo, Mardaraz, Pitta-Alvarez, & Giulietti, (1996), the presence of surfactant on blood agar plates results in the lysis of erythrocytes/hemolysis. In the present study, 18 bacterial isolates were reported as positive for haemolytic assay while the remaining seven isolates were negative for lysis of erythrocytes in blood agar plates. Blood agar is an enriched nutrient medium that supports the growth of various microorganisms (Pacwa-Plociniczak et al., 2016). However the use of blood agar has some limitations. First, the method is not specific, as lytic enzymes can also lead to clearing zones. Second, hydrophobic substrates cannot be included as sole carbon source in this assay. Third, diffusion restriction of the surfactant can inhibit the formation of clearing zones (Elazzazy et al., 2015; Hassanshahian, 2014). Few workers (Hassanshahian, 2014; Pacwa-Plociniczak et al., 2014; Yousuf et al., 2005) reported that some biosurfactants do not show any hemolytic activity at all in haemolysis assay Similarly, Youssef et al. (2007) also proved the poor relevance of this methodology.

Out of the 18 selected bacterial isolates, six were Gram negative and twelve were Gram positive in characteristic. These endophytes were detected as RE1 to RE6 (Gram positive) and RE11 to RE17, RE19, RE20, RE22-RE24 for Gram positive. Isolate RE1, RE5, RE6, RE12, RE14, RE17, RE19 and RE20 showed biggest haemolytic zone and selected for BATH and CTAB assay. Mulligan, Cooper, and Neufeld (1984) recommend the blood agar method as a preliminary screening method which should be supported by other techniques based on surface activity measurements. Hence, in the present investigation BATH assay, drop collapse test, oil spreading assay and emulsification assay were included to confirm biosurfactant production.

BATH Assay

The method relies on the degree of adherence of cells to various liquid hydrocarbons. The BATH assay is an easy but an indirect screening technique for biosurfactant production. Pruthi and Cameotra (1997) showed that the potential of bacterium to stick to hydrocarbons may be a characteristic feature of biosurfactant productive microbes. Bacterial adherence to hydrocarbons (*BATH*) *assay* was conducted with RE1, RE5, RE6, RE12, RE14, RE17, RE19 and RE20 endophytic isolates. Experimental results revealed that cell adherence with hexane ranged from 09.2±1.1 to 67.5±0.32%, and crude oil from 25.8±0.84% to 86.2±1.5%. Isolate RE1, RE6 and RE17 showed highest cell attachment with hexane (58.2±0.1%, 67.5±0.32%, and 52.2±1.8% respectively) and crude oil (74.66±1.4%, 86.2±1.5%, and 69.1±0.26% respectively).

CTAB Assay

This method is a semi-quantitative assay for biosurfactant detection (Palacios,

Gomez-Anduro, Bashan, & de-Bashan, 2016). The results revealed that all isolates were positive and showed a dark blue clear zone around the colonies. Isolates RE1, RE6 and RE17 developed 65 mm, 72 mm and 60 mm zone respectively, while RE5 and RE12 generated zones of 35 mm and 51 mm in size respectively. For the biosurfactant production and heavy metal removal studies, RE1, RE6, and RE17 were selected due to their effective results for Haemolytic assay, BATH assay and CTAB assay.

Biosurfactant Poduction

All three isolates, RE1, RE6, and RE17 were grown in glycerol supplemented medium for the production of biosurfactant. The cell-free broth was obtained and used for drop collapsing assay. The drop collapse methodology has been applied as a qualitative technique to discover biosurfactant-producing microorganisms in natural environments (Bodour et al., 2003). This assay is fast, simple to perform, reproducible and needs very little specialised instrumentation. RE1 and RE17 were positive for drop collapse assay. Isolate RE6 was negative for drop collapse activity while it was positive for hemolytic, BATH and CTAB assay. These results suggest that RE1 and RE17 produced extracellular biosurfactant and were reported positive for drop collapse test but RE6 was unable to produce extracellular biosurfactant. However, its cell acted as biosurfactant and showed positive results in hemolytic, BATH and CTAB assay. These results also suggested that in order to investigate the biosurfactant activity by microbial isolates, cell-free broth must be used instead of using cell culture (Karnwal, 2017).

Oil-Spreading Assay

Oil spreading assay results were in corroboration with drop collapse assay results. Isolates found positive with drop collapse test were also positive for oil spreading test. The oil spreading assay was utilised to review the surface activities of pure biosurfactant. This assay is fast and is extremely sensitive to surface active compounds (Ianieva, 2013). As for drop collapse test, RE1 and RE17 were shown to be positive, with similar results obtained for the oil spreading assay. Both RE1 and RE17 isolates showed high oil spreading activity due to extracellular biosurfactant production while isolate RE6 showed no oil spreading activity for present assay.

Emulsification index (E24) Assay

Emulsification properties of biosurfactants were measured in terms of emulsification activity (EA) and emulsification stability. The E24 assay is used to screen biosurfactant production by bacterial isolates (Jiménez et al., 2014). The presence of biosurfactant in cell-free broth will show emulsification of hydrocarbon in the test solution (Hajfarajollah, Mokhtarani, and Noghabi, 2014). In the present study, two hydrocarbons (crude oil and hexane) were used as the hydrophobic substrate.

Results revealed that cell-free broth of RE17 showed maximum %E24 value 77.5 with crude oil and 65.3 with hexane. The cell-free culture of RE1 showed a significant %E24 value with crude oil (69.9) and hexane (66.5). Result with RE6 was negative in comparison to other two isolates. Isolate RE6 did not show any %E24 with any hydrocarbon and reported negative for E24 assay. Emulsification activity is the measurement of a surface-active agent to make emulsions underneath bound conditions and directly associated with the oil drop size, the smaller of the dimensions was the higher of the activity (Abouseoud, Yataghene, Amrane, & Maachi, 2008; Ebrahimi & Tashi, 2012). The emulsion was produced as soon as one of the fluid phases was dispersed because of dispersal of tiny droplets in another liquid phase (Jiménez et al., 2014; Karnwal, 2017).

Hemolytic and BATH assays are not very reliable methods to test the biosurfactant production. Hence, it is inferred that extracellular products other than biosurfactants are responsible for the positive hemolytic and BATH activity observed with the strains showing negative emulsification activity (Thavasi, Sharma, & Jayalakshmi, 2011).

Effect of Temperature and pH on Heavy Metal Biosorption

The major advantages of biosorption technology are its effectiveness in reducing the concentration of heavy metal ions to very low levels and the use of inexpensive biosorbent materials. Many investigators have reported the biosorption of heavy metals into pure cultures of bacteria and algae and onto natural microbial populations as the new bioremediation technology (Gutnick & Bach, 2005). In the present study, bacterial isolates were introduced to the biosorption experiments. Optimisation of biosorption process by *Pseudomonas fluorescence* endophytes was studied on zinc, nickel, cadmium, and chromium in this investigation with pH, metal ion concentration, and temperature optimisation.

Temperature and pH have been considered as the most important factors influencing the biosorption process in aqueous solution (Liu, Liu, Ju, Li, & Yu, 2016; Shaaban, Ibrahim, Abouhend, & El-Moselhy, 2015). Change in temperature and pH influenced the dissociation of the active group on biosorbent but also interfered in the solution ion chemistry (Shaaban et al., 2015). In the present study, both isolates were incubated at different temperatures in heavy metal enriched medium at optimum pH. Results of temperature study revealed that maximum biosorption of heavy metal was reported at 32±1°C, however, at higher temperature, biosorption of heavy metal and microbial growth was decreased (Figure 2, Figure 3). Bacterial isolate RE1 showed 89%, 90%, 86% and 94% significant metal removal efficiency from the sample at 32±1°C for Zn, Cr, Cd and Ni, respectively. Similar results were observed with RE17 isolate with higher metal removal efficiency as

shown in Figure 5. The influence of pH on the biosorption capacity for the different metals is shown in Figure 2, 3, 4, and 5. Isolate RE1 and RE17 showed maximum metal removal efficiency at pH 7.5 for Zn and Ni (Figure 4, Figure 5). Meanwhile, maximum metal removal efficiency for Cr and Cd were recorded at pH 8 with 91% and 86% for RE1 and 92% and 98% for RE17, respectively.

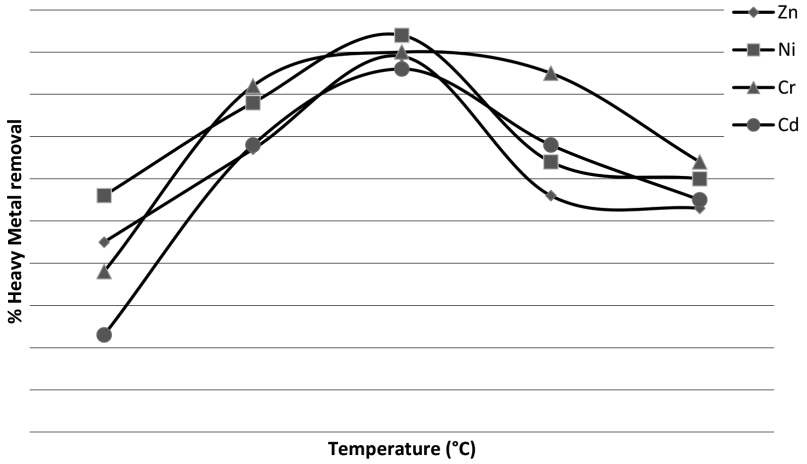


Figure 2. Percentage of heavy metal removal for isolate RE1 at various temperatures

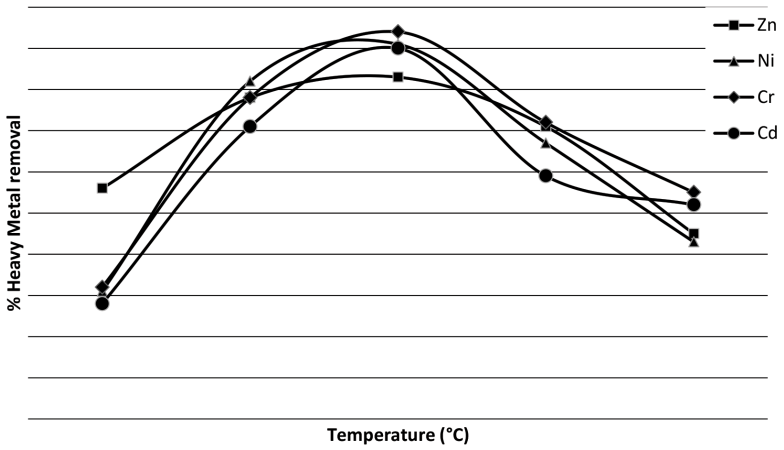


Figure 3. Percentage of heavy metal removal for isolate RE17 at various temperatures

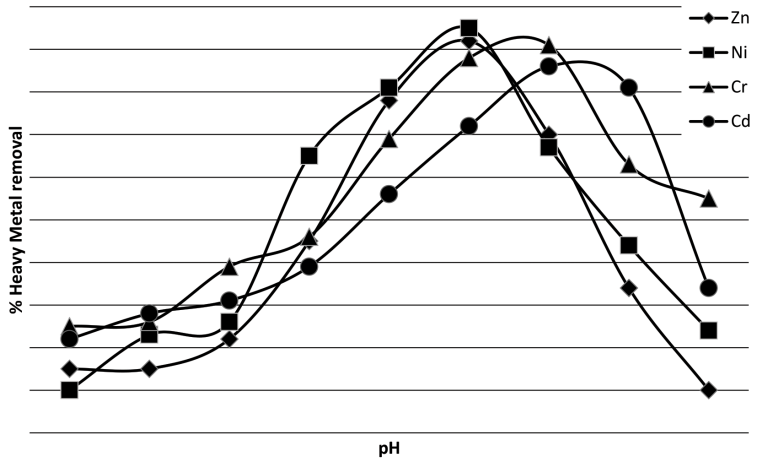


Figure 4. Percentage of heavy metal removal for isolate RE1 at various pH

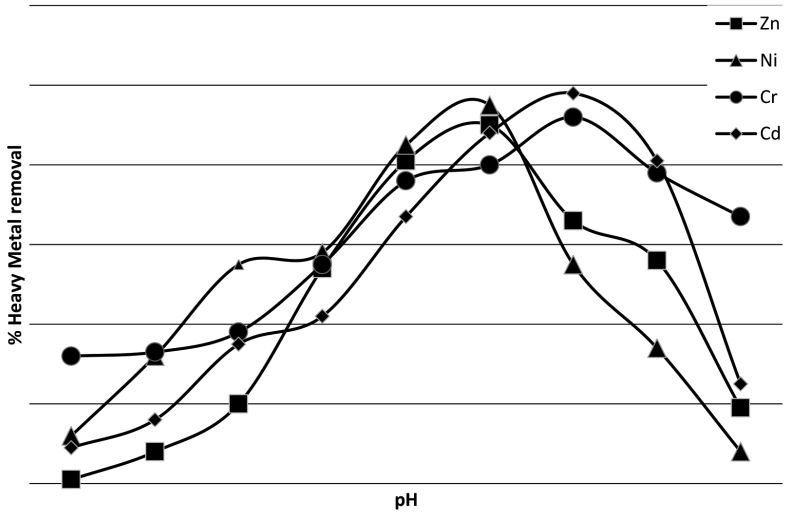
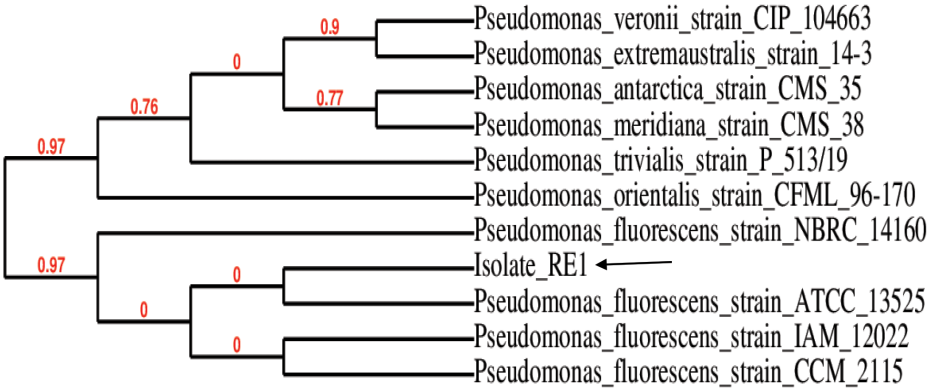


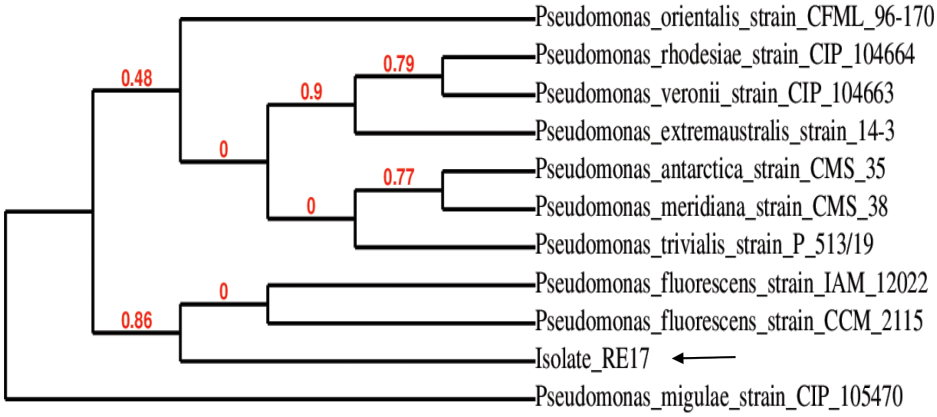
Figure 5. Percentage of heavy metal removal for isolate RE17 at various pH

16s rRNA Sequencing

BLAST analysis of the 16S rRNA gene sequence of RE1 and RE17 isolates demonstrated maximum sequence similarity with *pseudomonas fluorescens* strain ATCC 13525 (99%, Genbank Sequence ID: NR_114476.1 identical) and *pseudomonas fluorescens* strain CCM 2115 (98%, Genbank Sequence ID: NR_115715.1) respectively as shown in phylogenetic tree analysis by using MUSCLE alignment algorithm and TreeDyn phylogenetic tree building software (Figure 6).



(a)



(b)

Figure 6. Phylogenetic tree of bacterial isolates created by using TreeDyn, tree rendering software based on MUSCLE alignment data (a) BLAST similarity search results and phylogenetic tree for isolate RE1, (b) phylogenetic tree for isolate RE17

CONCLUSIONS

Heavy metals are toxic and hazardous to humans, marine life and the water body in which they are contained. The metals studied in this work included Zn, Ni, Cr and Cd that are known to show high toxicity for biological systems in the environment. Microbes play a vital role in the biosorption of heavy metals. The present study demonstrated the use of the endophytes *Pseudomonas fluorescence* isolated from rice, to remove heavy metals *in vitro*. It is recommended that further research be done to establish the specific mechanism involved in the biosorption processes.

REFERENCES

Abouseoud, M., Yataghene, A., Amrane, A., & Maachi, R. (2008). Biosurfactant production by free and alginate entrapped cells of pseudomonas fluorescens. *Journal of Industrial Microbiology and Biotechnology, 35*(11), 1303-1308.

Amer, R. A., Mapelli, F., El Gendi, H. M., Barbato, M., Goda, D. A., Corsini, A., ... & Abdel-Fattah, Y. R. (2015). Bacterial Diversity and Bioremediation Potential of the Highly Contaminated Marine Sediments at El-Max District (Egypt, Mediterranean Sea). *BioMed Research International*, *2015*, 981829. doi: 10.1155/2015/981829.

Bodour, A. A., Dreesand, K. P., & Maier, R. M. (2003). Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid Southwestern soils. *Applied and Environmental Microbiology*, *69*(6), 3280-3287.

Carrillo, P. G., Mardaraz, C., Pitta-Alvarez, S. J., & Giulietti, A. M. (1996). Isolation and selection of biosurfactant-producing bacteria. *World Journal of Microbiology and Biotechnology, 12*(1), 82-84.

Chen, X., Liu, X., Zhang, X., Caoand, L., & Hu, X. (2017). Phytoremediation effect of Scirpus triqueter inoculated plant-growth-promoting bacteria (PGPB) on different fractions of pyrene and Ni in co-contaminated soils. *Journal of Hazardous* Materials, *325*, 319-326.

Ebrahimi, A., & Tashi, N. (2012). Isolation of biosurfactant producing bacteria from poultry breast skin. *Jundishapur Journal of Natural Pharmaceutical Products, 7*(3), 93-96.

Elazzazy, A. M., Abdelmoneimand, T. S., & Almaghrabi, O. A. (2015). Isolation and characterization of biosurfactant production under extreme environmental conditions by alkali-halo-thermophilic bacteria from Saudi Arabia. *Saudi Journal of Biological Sciences*, *22*(4), 466-475.

Govarthanan, M., Mythili, R., Selvankumar, T., Kamala-Kannan, S., Rajasekar, A., & Chang, Y. C. (2016). Bioremediation of heavy metals using an endophytic bacterium *paenibacillus* sp. RM isolated from the roots of *tridax procumbens*. *3 Biotech, 6*(2), 242.

Gutnick, D. L., & Bach, H. (2005). Engineering bacterial biopolymers for the biosorption of heavy metals: New products and novel formulation. *Applied Microbiology and Biotechnology, 54*(4), 45l-460.

Hajfarajollah, H., Mokhtarani, B., & Noghabi, K. A. (2014). Newly antibacterial and antiadhesive lipopeptide biosurfactant secreted by a probiotic strain, propionibacterium freudenreichii. *Applied Biochemistry and Biotechnology, 174*(8), 2725-2740.

Han, J. I., Choi, H. K., Lee, S. W., Orwin, P. M., Kim, J., LaRoe, S. L., ... & Hur, C. G. (2011). Complete genome sequence of the metabolically versatile plant growth-promoting endophyte *Variovorax paradoxus* S110. *Journal of Bacteriology*, *193*(5), 1183-1190.

Hassanshahian, M. (2014). Isolation and characterization of biosurfactant producing bacteria from Persian Gulf (Bushehr provenance). *Marine Pollution* Bulletin, *86*(1-2), 361-366.

Ianieva, O. D. (2013). Biosurfactant-producing yeasts isolated from flowering plants and bees. *Mikrobiolohichnyi Zhurnal*, *75*(4), 53-59.

Jain, D. K., Collins-Thompson, D. L., Lee, H., & Trevors, J. T. (1991). A drop-collapsing test for screening surfactant-producing microorganisms. *Journal of Microbiological Methods, 13*(4), 271-279.

Ji, X., Lu, G., Gai, Y., Gao, H., Lu, B., Kong, L., et al. (2010). Colonization of morus alba L. by the plant-growth-promoting and antagonistic bacterium burkholderia cepacia strain Lu10-1. *BMC Microbiology, 10*(1), 243.

Jiménez, J. T., Barrera, M. A. R., Lucena, M. V., Flores, A. B., Segura, D., Corral, V. I. W., et al. (2014). Production of biosurfactants by bacteria isolated from a mine tailing zone in Southern Mexico and their resistance to heavy metals. *Journal of Bacteriology Research, 6*(4), 23-31.

Juwarkar, A. A., Nair, A., Dubey, K. V., Singhand, S. K., & Devotta, S. (2007). Biosurfactant technology for remediation of cadmium and lead contaminated soils. *Chemosphere*, *68*(10), 1996-2002.

Kamala-Kannan, S., & Krishnamoorthy, R. (2006). Isolation of mercury resistant bacteria and influence of abiotic factors on bioavailability of mercury - A case study in Pulicat Lake north of Chennai, south east India. *Science of the Total Environment, 367*(1), 341-352.

Karnwal, A. (2009). Production of indole acetic acid by fluorescent pseudomonas in the presence of L-tryptophan and rice root exudates. *Journal of Plant Pathology, 19*(1), 61-63.

Karnwal, A. (2017). Biosurfactant production by kcuria rosea and athrobacter luteolus using sugar cane waste as substrate. *II Ponte, 73*(2), 20-29.

Kassab, D. M., & Roane, T. M. (2006). Differential responses of a mine tailings *Pseudomonas* isolate to cadmium and lead exposures. *Biodegradation*, *17*(4), 379-387.

Khan, N., & Bano, A. (2016). Role of plant growth promoting rhizobacteria and Ag-nano particle in the bioremediation of heavy metals and maize growth under municipal wastewater irrigation. *International Journal of Phytoremediation*, *18*(3), 211-221.

Liu, B., Liu, J., Ju, M., Li, X., & Yu, Q. (2016). Purification and characterization of biosurfactant produced by bacillus licheniformis Y-1 and its application in remediation of petroleum contaminated soil. *Marine Pollution Bulletin, 107*(1), 46-51.

Liu, J., Liu, S., Sun, K., Sheng, Y., Gu, Y., & Gao, Y. (2014). Colonization on root surface by a phenanthrene-degrading endophytic bacterium and its application for reducing plant phenanthrene contamination. *PLoS One, 9*(9), e108249.

Morikawa, M., Diado, H., Takao, T., Murata, S., Shimonishi, Y., & Imanaka, T. (1993). A new lipopeptide biosurfactant produced by arthrobacter sp. strain MIS38. *Journal of Bacteriology, 175*(20), 6459-6466.

Morikawa, M., Hirata, Y., & Imanaka, T. (2000). A study on the structure function relationship of lipopeptide biosurfactants. *Biochimica et Biophysica Acta, 1488*(3), 211-218.

Mulligan, C., Cooper, D., & Neufeld, R. (1984). Selection of microbes producing biosurfactants in media without hydrocarbons. *Journal of Fermentation Technology, 62*(4), 311-314.

Palacios, O. A., Gomez-Anduro, G., Bashan, Y., & de-Bashan, L. E. (2016). Tryptophan, thiamine and indole-3-acetic acid exchange between chlorella sorokiniana and the plant growth-promoting bacterium azospirillum brasilense. *FEMS Microbiology Ecology, 92*(6), fiw077. doi:10.1093/femsec/fiw077

Pruthi, V., & Cameotra, S. (1997). Rapid identification of biosurfact ant-producing bacterial strains using a cell surface hydrophobicity technique. *Biotechnology Techniques, 11*(9), 671-674.

Rosenberg, M., Gutnick, D., & Rosenberg, E. (1980). Adherence of bacteria to hydrocarbons a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiology Letter, 9*(1), 29-33.

Sarubbo, L. A., Luna, J. M., & de Campos-Takaki, G. M. (2006). Production and stability studies of the bioemulsifier obtained from a new strain of candida glabrata UCP 1002. *Electronic Journal of Biotechnology, 9*(4), 400-406.

Shaaban, M. T., Ibrahim, H. A. H., Abouhend, A. S., & El-Moselhy, K. M. (2015). Removal of heavy metals from aqueous solutions using multi-metals and antibiotics resistant bacterium isolated from the Red Sea, Egypt. *American Journal of Microbiological Research, 3*(3), 93-106.

Shaharoona, B., Arshad, M., & Zahir, Z. A. (2006). Effect of plant growth promoting rhizobacteria containing ACC-deaminase on maize (zea mays L.) growth under axenic conditions and on nodulation in mung bean (vigna radiata L.). *Letters in Applied Microbiology, 42*(2), 155-159.

Siegmund, I., & Wagner, F. (1991). New method for detecting rhamnolipids excreted by pseudomonas species during growth on mineral agar. *Biotechnology Techniques, 5*(4), 265-268.

Srinivasan, R., Karaoz, U., Volegova, M., MacKichan, J., & Kato-Maeda, M. (2015). Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. *PLoS One, 10*(2), e0117617. doi:10.1371/journal.pone.0117617

Thavasi, R., Sharma, S., & Jayalakshmi, S. (2011). Evaluation of screening methods for the isolation of biosurfactant producing marine bacteria. *Journal of Petroleum and Environmental Biotechnology, S1*, 1-6. doi:10.4172/2157-7463.S1-001

Van der Vegt, W., Van der Mei, H., & Noordmans, J. (1991). Assessment of bacterial biosurfactant production through axisymmetrical drop shape-analysis by profile. *Applied Microbiology and Biotechnology, 35*(6), 766-770.

Youssef, N. H., Duncan, K. E., Nagle, D. P., Savage, K. N., Knapp, R. M., & McInerney, M. J. (2004). Comparison of methods to detect biosurfactant production by diverse microorganisms. *Journal of Microbiological Methods, 56*(3), 339-347.