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SCREENING OF RHAMNOLIPID PRODUCED BY MARINE BACTERIUM FOR HEAVY METAL REMOVAL IN MANGROVE SOIL

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Abstract: Mangrove located near urban area is exposed to various industrial discharge including heavy metals. Mangrove soil is capable of accumulating and storing these heavy metals. Heavy metals are toxic and non-biodegradable, so their accumulations affect water quality, while bioaccumulation and bio-assimilation of heavy metals in mangrove organisms negatively impact the food chain. Bacteria-derived biosurfactants are compounds capable of removing heavy metals from soil and sediment. Furthermore, environmentally friendly properties, such as biodegradability and low toxicity, exhibited by biosurfactants make them a suitable replacement for chemical surfactants for remediation efforts. This study was conducted to investigate the lead- (Pb) and zinc- (Zn) removing capability of rhamnolipid (RL), a type of biosurfactant produced by marine bacterium, *Pseudomonas aeruginosa* UMTKB-5. Rhamnolipid solutions of three different concentrations (25 mg/L, 50 mg/L and 75 mg/L) were added to mangrove soil and incubated for 7 days. The removal of Pb from soils was up to 18.3% using 25 mg/L RL solution, while 50 mg/L RL solution removed 48.3%, and 75 mg/L RL solution removed 75.9% Pb over time. Meanwhile, zinc removal of 25 mg/L RL solution was up to 24.9%, while 50 mg/L removed 16.5%, and 75 mg/L RL removed 30.5% of Zn. The results showed that RL from *P. aeruginosa* UMTKB-5 could be a potential biomaterial to be used to remediate heavy metals in sediment.

Keywords: biosurfactant, rhamnolipid, heavy metal, remediation, *Pseudomonas aeruginosa*

Introduction

Environmental contamination by heavy metals is largely known to be caused by rapid industrialization, urbanization and most anthropogenic actions, such as burning fossil fuels, mining, smelting, using pesticides, as well as discharging wastes from automotive exhaust, agricultural and domestic activities (Wuana & Okieimen, 2011). These released heavy metals eventually accumulate in soils. Soil and sediment are known to be sinks for the accumulation of heavy metals, especially mangrove sediment typically located near anthropogenic areas (Juwarkar *et al.*, 2007).

Despite occurring at seemingly minuscule environmental amounts, the accumulation of heavy metals affects water quality, endangers wild and agricultural flora as well as fauna through bioaccumulation, thereby harming organisms at higher trophic levels of the food chain via biomagnification, especially humans (Juwarkar *et al.*, 2007). By means of ingestion, inhalation and dermal contact, humans' exposure to heavy metals poses detrimental medical conditions, which include skin lesions, cancer, birth defects, organ damage, as well as retardation of physical and mental health (Singh & Cameotra, 2004). In the current era of rapid urbanization and industrial development, environmental pollution has been a major concern increasingly receiving public awareness. Such awareness has given rise to the call for the restoration or remediation of metal-contaminated environments. Since heavy metals cannot be easily biodegraded, research has steered towards conversion of the redox state of harmful heavy metals into a precipitated or volatilized, less toxic form (Singh & Cameotra., 2004).

Biosurfactants, a type of natural biologically-synthesised surface-active compound, are known to form ionic bonds with toxic heavy metals, which generate non-ionic metal–biosurfactant complexes with stronger stability thus less toxicity (Rufino *et al.*, 2012). Microorganisms produce biosurfactants as one of their metabolites because they constantly evolve their mechanisms for survival by adapting to or utilizing heavy metals for fundamental physiological functions, such as motility, biofilm formation, oil metabolization, cellular energy production and enzymatic reactions (Singh *et al.*, 2007; Gadd, 2010). Common microbial biosurfactants include emulsan by *Acinetobacter calcoaceticus* (Shoham *et al.*, 1983), sophorolipids by *Candida bombicola* (Kurtzman *et al.*, 2010), and rhamnolipid (RL) by *Pseudomonas aeruginosa* (Azemi *et al.*, 2016).

The biosurfactant known as RL, biosynthesised using *P. aeruginosa*, is an imperative biotechnological product with a wide range of applications. Due to its emulsifying, antiadhesive, antimicrobial and antifungal properties to name a few, RL possesses commercial prospects in biomedicine, cosmetics, food industry, agriculture, and heavy metal remediation (Mulligan *et al.*, 2001). Biosurfactants are preferred over synthetic surfactants especially in environmental industries, as biosurfactants have been reported for their diversity, low toxicity, biodegradability, potential for large-scale commercial production, broader metal selectivity, broader tolerance to pH, salinity and temperature, as well as producibility from by-product or industrial waste utilisation (Makkar *et al.*, 2011; Sriram *et al.*, 2011; Marchant & Banat, 2012).

Moreover, studies on the production of biosurfactants using marine bacteria have not been extensively done in Malaysia. Also, studies on heavy metal removal by bacteria will certainly provide a novel finding in Malaysia. Furthermore, the current study will set a precedent for using marine bacteria to produce biosurfactant to remediate heavy metals in soil. Therefore, in the present study, the bioremediation ability of RL biosynthesised from isolated marine bacterium *P. aeruginosa* UMTKB-5 in reducing heavy metals from mangrove sediment was investigated.

Materials and Methods

Strain Maintenance and Preparation of Inoculum

P. aeruginosa UMTKB-5 (GenBank accession number KT194193.1), a marine sediment isolate, was used in this study (Azemi *et al.*, 2016). Pre-culture was followed by activation phase before biosynthesis process to ensure adaptation of bacteria from solid to liquid medium for the preparation of inoculum. Two loopfuls of bacteria were transferred into 250-mL conical flask containing 50 mL nutrient rich broth and placed in an incubator shaker, *Ecotron CH-4103* (INFORS HT, Switzerland) then incubated for 12 h (200 rpm, 30°C). The steps were repeated to conduct second activation (Rahman *et al.*, 2003; Christova *et al.*, 2013).

Biosynthesis of RL

Mineral salt medium (MSM) used for biosynthesis of RL comprised of 2.80 g/L potassium dihydrogen phosphate (KH_2PO_4) (R&M, Malaysia), 3.3 g/L disodium hydrogen phosphate (Na_2HPO_4) (R&M, Malaysia) and 0.25 g/L of urea ($CO(NH_2)_2$) (Merck, USA) (Azemi *et al.*, 2016). MSM was also supplemented by 1 mL/L trace elements solution, 1 mL/L of 0.25 g/L magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), 20 g/L of glycerol and 7% (v/v) bacterial cells of pre-culture medium (Christova *et al.*, 2013, Azemi *et al.*, 2016). Then, the medium was incubated using *Ecotron CH-4103* (INFORS HT, Switzerland) for 72 h (200 rpm, 30°C) (Azemi *et al.*, 2016). The cultivation was done in triplicates in a final medium volume of 200 mL in 1L Erlenmeyer flask.

Cell Harvesting and Separation of Supernatant

After 72 h of biosynthesis, the medium was subjected to a centrifugation speed of 8000 rpm for 5 min at 4°C for separation of cell pellet and supernatant (Yin *et al.*, 2009). The cell pellets were subjected to oven drying at a temperature of 105˚C until the cell dry weight (CDW) achieved was consistent. Meanwhile, supernatant was stored in 4°C chiller, 2D-DC-GE (JUSCOOL, Malaysia) until use for quantification of RL and subsequent extraction of RL.

RL Quantification

The RL content in supernatant was quantified using Orcinol assay technique (Ballot, 2009). In a microcentrifuge tube, 750 µL diethyl ether (AR Grade) was utilized to extract 400 µL of RL-containing supernatant. The microcentrifuge tube was vortexed for 3 min to obtain the upper ether fraction which was transferred into another microcentrifuge tube. The extraction process was repeated twice and the microcentrifuge tube containing upper fraction was left overnight to be dried. The following steps proceeded with the addition of 400 µL phosphate buffer solution (PBS) (pH 8) and the mixing of 900 µL Orcinol assay with 100 µL PBS-added mixture, which was afterwards placed in a water bath for 30 min at 80°C (Azemi *et al.*, 2016). Orcinol assay was prepared as described by Azemi *et al.* (2016). Before obtaining the absorbance reading at 421 nm, the solution was left in a cupboard for 35 min (Ballot, 2009; Abdel-Mawgoud *et al.*, 2011). The concentration was multiplied by 2.25 as correction factor to account for the relative proportion of congeners of RL and the lipid portion (Déziel *et al.*, 2000).

RL Extraction

The RL extraction from supernatant was done according to a study by Yin *et al.* (2009). Supernatant was acidified to pH 2 using 6M hydrogen chloride (HCl). Later, in a separating funnel, ethyl acetate and supernatant were added at 1:1 ratio, and the separating funnel was shaken vigorously for about 3 min. The funnel was left aside until phase separation occurred where the upper fraction containing RL was taken. This step was repeated twice and, to remove excess water, the upper fraction was transferred into conical flask where it was added with anhydrous sodium sulphate and left aside for about 30 min. After that, *Buchi 1234* (Buchi, Japan), a rotary evaporator, was used to extract the crude RL with a 40°C water bath. The 0.05M sodium bicarbonate was used to dissolve crude RL, after which 6M of HCl was used to tune the pH to pH 2. Then, a chiller of 4°C was used as 24-h storage for the solution. Subsequently, the solution was subjected to a 15-min centrifugation at 12500 rpm. Then, using *MDF-U537* (Sanyo, Japan) Biomedical Freezer, the RL precipitate was stored at -80°C overnight before *Freezone 4.5 Freezer Dry System* (Labconco, USA) was used to freeze-dry the precipitated RL for 24 h.

Sample Collection and Heavy Metal Analysis

Sampling was done in mangrove area in Universiti Malaysia Terengganu (UMT) due to proximity with urban development activities. Mangrove soil samples were collected in replicates using Ponar grab to obtain surface soil at approximately 10 cm depth. To ensure undisturbed grain-size distribution, precautionary steps were taken to reduce soil disturbance during sampling. Only samples that were collected with firmly-closed grab sampler and only inner soil was selected as acceptable for use. Sterile plastic containers and an approximate temperature of 4°C were provided for post-collection storage prior to further analysis (Azrina *et al.*, 2006). Then, digestion of soil samples was carried out to subsequently analyse the samples for total lead (Pb) and Zinc (Zn) (Defew *et al.*, 2005).

Sample that was finely powdered and weighed to 50 mg, as well as 1.5 mL mixed acid solution of concentrated hydrofluoric acid, nitric acid and HCl, were placed into a sealed Teflon vessel to be heated using the digestion method. The ratio of the acids was 1:3:3. The Teflon vessel was heated for 7 h at 100°C. After that,

the vessel was cooled until room temperature was achieved. A 10-mL polypropylene test tube was used for the content to be transferred into. Next, deionised water was used to dilute the content to 10 mL. At the last stage, a clear solution with absence of residue was acquired. Precise and quick determination of Pb and Zn in the digested soil was carried out using atomic absorption spectroscopy (AAS) (Yunus *et al.*, 2011). A precision threshold of less than 3% that was evaluated by the replicate analyses was considered as acceptable (Yunus *et al.,* 2011). Duplicate Canadian Certified Reference Material Project MESS-3 was analysed to examine the accuracy of the result that was within the certified value of ±3%.

Heavy Metal Removal

Soil heavy metal removal was done based on methods prescribed by da Rocha Junior *et al*., (2018) with some modifications. A total of 5 g of soil samples were transferred into wide mouth conical flasks and RL was added in different concentrations of 75mg/L, 50mg/L, and 25 mg/L. The experiment was done in triplicates for each concentration. Later, 0.05 g of soil was collected at selected interval for heavy metal analysis. The experiment was conducted for a total of 7 days.

Results

Table 1 shows RL production by *P. aeruginosa* UMTKB-5 when supplemented with glycerol as substrate. The cells were able to yield up to 1.8 g/L of RL concentration which translated to an estimated 2.5 g/L RL per gram of cells.

Table 1: Concentration and yield of RL biosynthesised by *Pseudomonas aeruginosa* UMTKB-5 utilising glycerol as a carbon source.

RL concentration [a] (g/L)	**Cell Dry Weight (CDW), (g/L)**	**Yield of RL $Y_{RL/CDW}$ [b] (g/g)**	**Yield of RL $Y_{RL/S}$ [c] (g/g)**
1.80 ± 0.03	0.42 ± 0.01	2.5 ± 0.2	0.05 ± 0.01

[a] RL concentration was determined using orcinol assay, [b] $Y_{RL/CDW}$, yield of per gram of RL per gram cell CDW, [c] $Y_{RL/S}$, Yield of per gram of RL per gram of substrate, data show the mean ± standard deviation of triplicates.

Figure 1 and 2 show Pb and Zn removal by three different concentrations of RL solutions respectively over a seven-day period. In general, both Pb and Zn were removed by all tested concentrations of RL. It was observed that 75 mg/L of RL solution removed higher amount of Pb and Zn compared to 50 mg/L and 25 mg/L of RL solution. However, the finding of this study revealed significant dissimilarity in the metal removal of Pb and Zn, where RL solutions were able to remove Pb better than Zn at all tested RL concentrations.

Results showed that 75 mg/L of RL solution was able to remove 82.45% of Pb compared to 30.50% of Zn which showed a 51.95% removal difference. Similar result was observed at 50 mg/L of RL solution where 63.07% of Pb was removed compared to 24.90% of Zn. Based on Figure 2, Zn removal was only recorded until day 4 for all test conditions and no further removal was observed on day 7.

Figure 1: Removal of Pb in mangrove soils from 1 to 7 days by different concentration of isolated Rhamnolipid (RL) at 75mg/L, 50mg/L and 25 mg/L RL solutions.

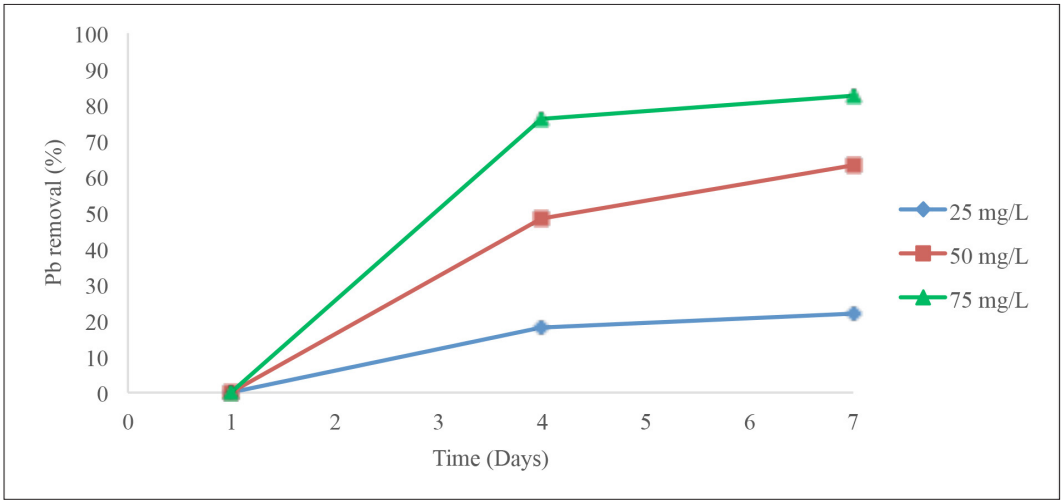
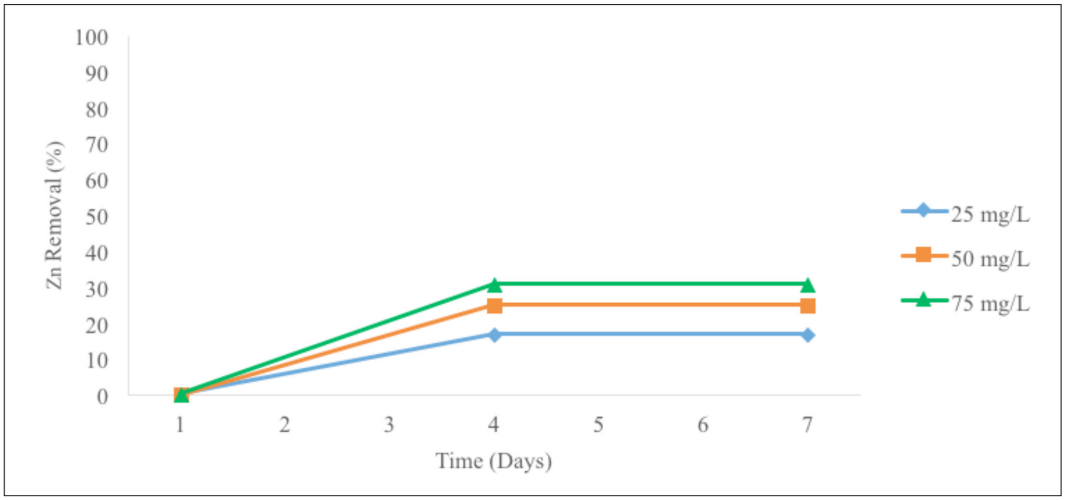


Figure 2: Removal of Zn in mangrove soils from 1 to 7 days by different concentration of isolated Rhamnolipid (RL) at 75mg/L, 50mg/L and 25 mg/L RL solutions.



Discussion

RL concentration produced in this study was approximately 17% higher than in a previous study reported by Rashid *et al*., (2015) where the concentration obtained was 1.49 ± 0.9 g/L. Both studies utilized the same carbon source and bacterial strain, which was glycerol (20 g/L) and *P. aeruginosa* UMTKB-5 respectively. However, different nitrogen sources were used, whereby $CO(NH_2)_2$ was used in this study and ammonium chloride (NH_4Cl) was used in the study by Rashid *et al.* (2015). The different nitrogen source may have caused the difference in the RL concentration. Similar result was reported in a previous study by Wei *et al* (2008), whereby high RL concentration was produced by *P. aeruginosa* J16 when supplemented with $CO(NH_2)_2$ as nitrogen source compared to NH_4Cl.

It has been reported that RL has different affinities for bonding with different metals (Mulligan, 2004; Dahrazma & Mulligan, 2007), in the following descending order: $Al^{3+} > Cu^{2+} > Pb^{2+} > Cd^{2+} > Zn^{2+} > Fe^{3+} > Hg^{2+} > Ca^{2+} > Co^{2+} > Ni^{2+} > Mn^{2+} > Mg^{2+} > K^{+}$ (Ochoa-Loza *et al.,* 2001). This shows that RL in soil can remove Pb and Zn from the sediment. A study was done by Wang and Mulligan (2009) on heavy metal removal with soil flushing, which included As, Cu, Pb, Zn and Fe. The RL-metal complexes had stability constants reported in the following order: $Cu^{2+} > Pb^{2+} > Zn^{2+} > Fe^{3+}$. The reported results corroborate the findings of the current study where Pb was removed at higher percentage compared to Zn. Natural-occurring organic ligands, such as humic or fluvic acids, and RL can form complexes with metals (Ochoa-Loza *et al.*, 2001). However, metals had a higher stability constant with RL compared to the organic ligands mentioned (Ochoa-Loza *et al.*, 2001). Thus, RL performs better in removing heavy metals despite other potential and naturally occurring elements that can bind with metals in the soil. Furthermore, a previous study by Neilson *et al.* (2003) primarily on removal of Pb by RL showed that 15% Pb was removed by 10 mM solution of RL.

However, heavy metals cannot be degraded by RL and form strong bonds with soil, causing difficulty in removal by RL (Singh & Cameotra, 2004). The efficiency of heavy metal removal by RL is dependent on soil composition and pH, particle size, capacity of cation exchange, type of contamination and the time of occurrence as well as geological layout (Mulligan *et al.*, 2001). RL biosynthesised by *P. aeruginosa* strains has been reported to form bonds with complex cationic metal species including Zn, Cd, and Pb. Furthermore, the RL from this strain is less likely to form complexes with normal soil metal cations of lower affinities, such as Mg and Ca, than toxic metals, such as Pb and Cd (Singh & Cameotra., 2004).

Mulligan *et al.* (2001) hypothesised that the removal of metals using biosurfactants is related to surfactant micelles through surfactant adsorption to surface of the soil, complex-forming with metal, and detachment of metal from soil hence into the solution. It is possible that the removal of Zn in mangrove soil remained unchanged from day 4 to 7 due to insufficient binding sites or low concentration of RL as approximately 2 moles of RL is required to bind to per mole of metal (Ochoa-Loza *et al.*2001). Thus, affinity and number of binding sites proportionally related to the concentration of RL (Ochoa-Loza *et al.,* 2001; Sandrin *et al.,*2000).

To completely remove heavy metals, Singh and Cameotra (2004) suggested that the soil be treated with biosurfactant using appropriate mixing method. The positive-charged metals and negative-charged surfactant form strong bonds sufficient for surfactant metal complexes to be removed by water-flushing, thereby leaving metal-free soil (Singh & Cameotra, 2004). The biosurfactant could be subjected to multiple recycling as the biosurfactant was not degraded by the recovery method (Wang & Mulligan, 2009). Consequently, the application potential of the RL biosynthesised by *P. aeruginosa* UMTKB-5 for heavy metal remediation process is highlighted by the findings of this study.

Conclusions

This study demonstrates that RL produced by the isolated marine bacterium *P. aeruginosa* UMTKB-5 has potential heavy metals removing ability when tested with mangrove soils. Due to the low toxicity of the biosurfactant, it is very promising for use in metal removal. It is recommended that further studies be conducted with the soil-washing technique where the biosurfactant-metal complex is flushed out and more RL is added within the time series.

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