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Research Article

Evaluation of the Impact of Crude and Purified Biosurfactants Synthesized by *Pseudomonas aeruginosa* in the Marine Environment

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Abstract

The evaluation of the impact of crude and purified biosurfactants synthesized by *Pseudomonas aeruginosa* strain NCIMB 8626 was examined. Crude and purified biosurfactants impacted the available Pb present in the sediment after the incubation period of 14 days substantially with 27.41% and 27.67% reduction respectively. Maximum concentration of (sediment + Pb) was about 16.39 ± 2.08 mg/g before incubation compared with after incubation of about 15.07 ± 1.87 mg/g. The results obtained shows no significant difference between crude and purified biosurfactants, thus, determining the potential and most effective forms of the biosurfactant could not be established. In addition, there was relative increase in microorganisms most especially bacteria in treatment samples containing crude and purified biosurfactants as most were too numerous to count. However, it appears that growth medium containing treatment samples showed a higher enumeration throughout and this may be attributed to additional nutrient sources provided by the growth medium itself. Uniquely, all the treatment samples showed significant increase in microbial population after the 14 days incubation periods compared to "before" incubation period.

Keywords: Bioremediation, lead, microorganisms, sediment, heavy metal, incubation periods.

Introduction

The steady redistribution of many harmful and toxic heavy metals from the earth's crust to the sediment is as a result of rapid developments and increasing mining and industrial activities. The likelihood of human exposure to these toxic heavy metals via inhalation, dermal contact or ingestion has been substantially raised. Heavy metals contaminated sediments most especially by lead (Pb) are environmentally a widespread problem and a priority (Khalef *et al.*, 2022). Pb is one of the most commonly encountered heavy metals in the sediment and it normally displays increased adsorption affinity on sediments. Pb contamination primary sources are from mining and smelting activities, engine leaded gasoline combustion, land uses of sewage sludge, disposal of battery and Pb containing products (Ahmad *et al.*, 2021), and requires mitigating technologies to curb its spread. According to Arora and Khosla, (2021) conventional technologies for treating Pb contaminated sediments have several inherent demerits due to the fact that these technologies cannot remove completely hazardous contaminants, but they can only stabilize hazardous heavy metals in the contaminated medium. However, some bacterial strains have been discovered to possess the ability to concentrate or bio-remediate heavy metals into forms that may be precipitated or volatilized. These forms may become less toxic and thus easily disposable (Jessica *et al.*, 2020). There are reports on heavy metals such as Pb, Cd, Zn, Cu etc. removal using surface active compounds such as biosurfactants (Aragão *et al.*, 2018; Sałek and Euston, 2019). In comparison with traditional physic-chemical methods, bioremediation technology does not aggravate other environmental problems but to clean-up the contaminated sediment partially or completely to its pristine state. Bioremediation depends on the natural sediment organisms or the augmentation of non-native organisms such as microorganisms and/or plants with special metal-binding capacity to decontaminate the heavy metal or reduce its toxicity through immobilization (Lwin *et al.*, 2018). The application of microbes and their microbial product such as biosurfactants to remove heavy metals as a technology

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have been successfully implemented in wastewater such as sewage sludge, industrial effluents, and mine water. The mechanisms employed in these systems take advantage of the microbial-metal interactions to bioconcentrate and separate heavy metals from the wastewater. These mechanisms (microbial-metal interactions) that have been described in many current reviews (Vineet, 2018; Banerjee *et al.*, 2018) include metal binding to the cell surface or within the cell wall, translocation of the metal into the cell, volatilization of the metal as a result of a biotransformation reaction, and the formation of metal precipitates by reaction with extracellular polymers or microbially produced anions such as sulphide or phosphate (Vineet, 2018). Thus, the aim of this study is to evaluate the impact of biosurfactants (crude and purified) produced by *Pseudomonas aeruginosa* strain NCIMB 8626 on the microbial populations (bacteria, actinomycete and fungi) adsorbed on sediment. The methods adopted include microbial culture, fermentation, isolation and extraction process.

Materials and Methods

Bacterial strain: Rhamnolipid biosurfactant was synthesized from a strain of *Pseudomonas aeruginosa* NCIMB 8626 for the purpose of this study.

Basal mineral salt medium: According to the description of Zhang *et al.* (2005) the basal mineral salt medium (BSM) composition used in g/L was: $NaNO_3$ = 4.0 (sodium nitrate) NaCl = 1.0 (sodium chloride) KCl = 1.0 (potassium chloride) $CaCl_2.2H_2O$ = 0.1 (calcium chloride dihydrate) KH_2PO_4 = 3.0 (Dihydrogen potassium phosphate or phosphoric acid) $Na_2HPO_4.12H_2O$ = 3.0 (Di-sodium hydrogen orthophosphate 12-hydrate) $MgSO_4$ = 0.2 (magnesium sulfate) $FeSO_4.7H_2O$ = 0.001 (Iron (II) sulfate or ferrous sulphate) Trace element stock solution = 2 mL

Bacterial preculture: *Pseudomonas aeruginosa was* maintained on BH broth and incubated at 37°C for 24 h with gyratory shaking at 150 rpm. The cells were washed twice in BSM by centrifuging culture broth at 3000 rpm for 10 minutes, and then resuspend in 40 mL of BSM with 3g/L glycerol added and left for 48 h to ensure that all alternative carbon sources have been completely utilized (Kubicki et al., 2020; Almeida *et al*, 2021).

Biosurfactant: *Pseudomonas aeruginosa NCIMB 8626 was used to produce rhamnolipid biosurfactant in the laboratory and used as biosurfactant for this research experiment.*

Biosurfactant fermentation: BSM of 250 mL was placed in a 1 L Erlenmeyer flask and inoculated with 5% inoculum of *P. aeruginosa* NCIMB 8626. 30 g/L of glycerol was added as carbon source (6 mL) and the flask was incubated at 37°C with gyratory shaking at 150 rpm for 5 days. Most rhamnolipid are found to accumulate at the stationary stage of cell growth.

Isolation procedure: The culture medium was centrifuged at 6000 rpm for 20 min, and the supernatant (pH 8.1) isolated and acidified with 5 mol/L H_2SO_4 to pH 2.0. The acidified supernatant was allowed to stand overnight at 6°C and followed by solvent extraction with the two-step Bligh and Dyer method using chloroform and methanol as solvent and Rhamnolipid was after the fermentation period of 5 days.

Rhamnolipid extraction: The extraction of rhamnolipid from culture supernatant was performed using the Bligh and Dyer method.

Rhamnolipid purification by column chromatography: The column (27 × 2 cm) was packed with silica gel (40-63 µm particle size for flash column chromatography) by the dry pack method and wetted with chloroform just to the top of the silica gel. Sample was dissolved in small amount of chloroform (1 mL) and loaded to the top of the column, and small portion of chloroform added and drained until the sample mixture was a little way into the adsorbent. The column was pre-eluted with chloroform to remove the yellow pigment associated with the crude rhamnolipid sample and to elute neutral (simple) lipids. The column was then run with chloroform: methanol mobile phase sequence: 10:1 v:v (250 mL); 1:1 v:v (300 mL) and 1:10 v:v (150 mL) and fractions collected.

Analytical method

Determination of Rhamnolipid concentration: Sample of about 200 µL was treated with 1.8 mL of a solution containing 0.19 % orcinol (in 53% H_2SO_4) and boiled in hot water bath for 20 minutes (Orcinol used was 5-methylresorcinol monohydrate). After cooling at room temperature for 15 minutes, sample was vortexed and absorbance measured with spectrophotometer at 421 nm. Standard curve was prepared with L-rhamnose (L (+)-rhamnose monohydrate from Acros) and the concentration of rhamnolipid estimated by comparing the data obtained for the unknown sample over the linear correlation demonstrated between the concentration of rhamnolipid and optical density with those of rhamnose standards measured between 0 and 100 µg/mL. The rhamnose values can be expressed as rhamnolipid values by multiplying them with a coefficient of 3.4 obtained from the correlation of pure rhamnolipid/ rhamnose (Sharma, 2018).

Test for toxicity of biosurfactants: Toxicity measurements of cultivated medium containing rhamnolipids produced by *P. aeruginosa* was carried out using Microtox®; a quick method for determination of toxicity of aquatic samples in

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terms of reduction in luminescence naturally emitted by the bacterium *vibrio fischeri*. The toxicity was measured in terms of the effective concentration (EC50) after 15 minutes.

Analysis of sediment sample: Physico-chemical parameters analyzed were as pH, sediment texture, moisture content, and organic matter, nitrate, phosphorus, and background check of heavy metal concentration were also examined. A microbial investigation of the sediment was also carried out using plate count technique.

Preparation of Sediment Sample: Upon arrival in the laboratory; the sediment was spread out and kept on the laboratory bench to air-dry at room temperature (28 ± 2°C) for 7 days. After air-drying, sediment was ground with mortar and pestle and passed through a <2 mm sieve. This <2 mm sieve air-dried sediment was then use to carry out the microbial and physico-chemical analyses.

Sediment pH: 10 g of air-dried sediment was weighed and poured into a 100 mL glass beaker, then 10 ml of deionized water was introduced and the mixture was stirred and allowed to stand for 30 minutes. Suspension was stirred at 10 minutes interval during this period. After 1 hour, the suspension was stirred and the combine electrode was placed in the suspension (about 3-cm deep) and the pH readings were recorded.

Sediment texture: About 40g of air-dried sediment sample was weighed into a 600 mL beaker and treated with 60 mL dispersing solution. The beaker was covered with a watch-glass and left overnight. Content was transferred quantitatively from the beaker to a sediment-stirring cup and filled with water to about three-quarters. Suspension was stirred at high speed for 3 min using the special stirrer. Stirring paddle was rinsed into a cup and allowed to stand for 1 minute and transferred quantitatively into a 1 L calibrated cylinder (hydrometer jar), and brought to volume with water. The cylinder was tightly closed with stopper and shake for several times to allow the sediment particles to disperse completely. The stopper was removed and hydrometer was immediately placed in the suspension as described by Gangwar and Baskar (2019). The first reading was taken exactly 40 seconds after placement of hydrometer. The cylinder was closed with the stopper and inverted several times again to ensure complete dispersal of particles. The hydrometer was placed in the suspension exactly after 4 hr and the second reading was noted. The blank was simultaneously run without sediment and the room temperature was recorded

Moisture content: All analysis in the laboratory was related to an air-dried basis, and therefore must consider the actual sediment moisture content (Peng *et al.*, 2018). About 10 g of air-dried sediment was weighed into a previously dried and weighed metal can with lid. Dry in an oven with lid unfitted, at 105°C overnight. Cooled in desiccator for 30 minutes and re-weighed.

Organic matter content: The loss by ignition method was used to carry out this analysis. An empty crucible was put into the muffle furnace and leftover night at 105°C. The crucible was then removed from the furnace and allowed to cool in the desiccators and weighed. Air-dried sediment sample was introduced into the crucible and the weight was recorded. The crucible and its content were placed in the muffle furnace and leftover night at 105°C after which its weight was determined when cooled. For this process, the sediment was analyzed in duplicate.

Nitrate extraction: About 25 ml of deionize (DI) water was added into sediment bottle containing 3.5 g of air-dried sediment and 1 shot Nitrate Extraction Powder was added to the bottle, capped and shook for 30 sec. The sediment coagulated in the bottom of the bottle leaving a clear extract. 1.0 ml of the aqueous sediment extract was pipette into a sample cell and filled up with DI water to the 25 ml mark. 1 NitriVer 6 Powder Pillow content was added to the cell, swirl stoppered and shake continuously for 2 min after which it was allowed to settle for 2 min. 25 ml of the sample was poured into another clean sample cell and the content of 1 NitriVer 3 Reagent Powder Pillow was added, stoppered and shook for 30 sec thereafter allowed to settle for 10 min. The same process was used for the blank but without the sediment sample. The Hash Spectrophotometer at 500 nm was used to take the readings.

Available Phosphorus: The available phosphorus was determined using a modified procedure of Watanabe and Olsen (1965). Phosphorus concentration in the sample was read from the calibration curve.

Background check of sediment heavy metals: Methodology devised by Carapeto and Purchase (2000) was used to determine the heavy metal (Pb) availability in the sediment using 1 g of sediment in 10 ml of EDTA. All the analyses were carried out in duplicates and the ICP generate three readings.

Microbial plate count of sediment: The plate count technique was used to analyze the enumeration of sediment micro-organisms. 1 g of sediment was weighed onto the sterile filter paper and was added to 99 mL of sterile water. The cap of the bottle was replaced and the solution was mixed by shaking for 3 minutes. Dilutions were made by transferring 1ml of the 10^{-2} (1:100) dilution to a second bottle of sterile water and mixed, this resulted in

*Corresponding author

the 10^{-4} dilution. About 1 mL of the 10^{-4} dilution was transferred to the final bottle of sterile water and mixed-resulting in 10^{-6} dilution. The Petri dishes were labeled as follows:
Tryptic Soya Agar (TSA) – Bacteria 10^{-4} to 10^{-7}
Glycerol Yeast Extract Agar (GYEA)–Actinomycetes 10^{-3} to 10^{-6}
Sabouraud Dextrose Agar (SDA) – Fungi 10^{-2} to 10^{-5}

Using aseptic techniques, the 1000 and 2000 µL pipette were used to transfer the proper amount of each sediment dilution to each of the Petri dishes with the appropriate label. The agar bottles were placed in a water bath from which they were removed and about 20 mL of each of the agars was poured into the appropriate dishes. The dishes were mixed thoroughly by swirling and then allowed to set. This was done for all the agars (TSA, GYEA and SDA). The plates were inverted and incubated at 25°C for 5 days after which the results were obtained by Plate counting between 25 and 250 colonies. This was used to determine the number of bacteria, actinomycetes and fungi present in 1 g of sediment.

Experimental design: The air-dried sediment was artificially contaminated with lead (Pb). Lead (Pb) (500 mg/kg of sediment) was applied as lead (II) acetate trihydrate (Pb $(CH_3COO)_2.3H_2O$) to sediment with or without biosurfactants (crude or purified) and *E. coli* as shown in Table 1. All mixings were performed thoroughly to ensure homogeneity (Fig. 1a-b).

Fig. 1a-b. Homogenous mixed (spiked) experimental sediment pots.

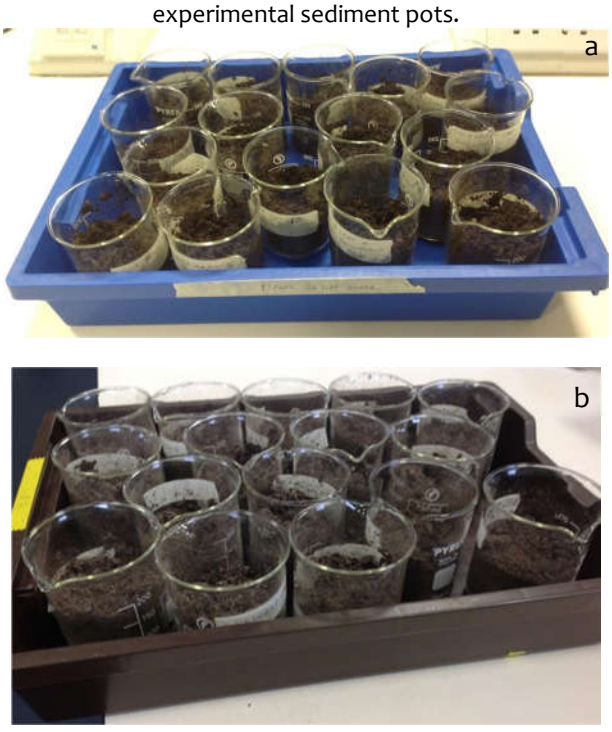

Table 1. Experimental design.

Control	Treatment 1	Treatment 2	Treatment 3
Sediment	Sediment + Crude Bio.	Sediment + Purified Bio	Sediment + *E. coli*
Sediment + Pb	Sediment + Crude Bio. + Pb	Sediment + Purified Bio + Pb	Sediment + *E. coli* + Pb

Results and Discussion

Table 2 shows the physico-chemical parameters of the sediment sample such as pH, sediment classification, texture, moisture content, organic matter content analyzed in the laboratory prior to the experiment. The parameters clearly show the characteristics of the sediment analyzed.

Table 2. Physico-chemical parameters of sediment before incubation.

Parameter	Sediment sample
pH	6.84
Classification	Loamy sand
Texture	Sand 80.12%; Clay 15.38%; Silt 4.50%
Moisture content	34.15%
Organic matter content	46.61%
Soil heavy metals background check Pb	0.15 ± 0.21 mg/g

Table 3 shows different microorganism counts of sediment sample before and after incubation in colony forming unity per gram. The microorganisms observed were bacteria, actinomycetes and fungi. The medium with abundant bacteria were found in (D = sediment + Crude Biosurfactant + Pb), (E = Sediment + Purified Biosurfactant), (G = Sediment + Growth Medium), (H = Sediment + Growth Medium + Pb). The sediment sample pH was 6.84 and this was relatively stable throughout the course of this study. Similarly, in the study conducted by Roberts (1998) it was reported that the solubility, mobility and ionized forms of heavy metals most especially Pb is significantly influenced by the sediment pH. The moisture content and organic content of the sediment sample were 34.15% and 46.61% respectively while the sediment type was loamy sand which was made up of 80.12% sand, 15.38% clay and 4.5% silt as shown in Table 2. Table 4 shows the concentrations of available Pb in experimental treatment pots before and after incubation. The different treatment pots indicate fluctuation in the values. The highest value of about 16.39 ± 2.08 mg/g was dictated in "before test" and the lowest recorded as 3.49 ± 0.09 mg/g. After incubation, highest value obtained was 15.07 ± 1.87 mg/g, while the lowest value was about 3.05 ± 0.49 mg/g.

*Corresponding author

Table 3. Microorganisms' enumeration of sediment sample before and after incubation in colony forming unity per gram (CFU/g).

	Total mean							
	Before incubation CFU/g x 10^5 ($\bar{x}$ ± SD)				After incubation CFU/g x 10^5 ($\bar{x}$ ± SD)			
Microorganisms	A	B	C	D	E	F	G	H
Bacteria	4.75 ± 0.35	TNC	10.34 ± 0.17	21.21 ± 0.35	TNC	TNC	14.60 ± 0.56	TNC	TNC
Actinomycetes	4.85 ± 0.91	20.15 ± 0.17	9.37 ± 0.21	16.20 ± 0.20	15.85 ± 0.66	TNC	17.40 ± 0.14	28.95 ± 0.64	TNC
Fungi	12.50 ± 3.53	15.33 ± 0.88	7.05 ± 0.27	9.30 ± 0.42	9.91 ± 0.17	14.55 ± 0.49	10.70 ± 0.56	14.55 ± 0.59	13.35 ± 0.49

Identification:
A = Sediment
B = Sediment + Pb
C = Sediment + Crude Biosurfactant
D = Sediment + Crude Biosurfactant + Pb
E = Sediment + Purified Biosurfactant
F = Sediment + Purified Biosurfactant + Pb
G = Sediment + Growth Medium
H = Sediment + Growth Medium + Pb
TNC = Too Numerous To Count
$\bar{x}$ ± SD = Mean ± Standard deviation of duplicates
Microorganism = Colonies number x Dilution factor/Weight of dry soil.

It was observed that fungi were the most dominant group in the sediment sample prior to spiking and before incubation with a total mean colony count of 12.5 0 ± 0.35 (CFU/gx10^5) which was immediately followed by Actinomycetes and Bacteria with 4.85 ± 0.92 and 4.75 ± 0.35 (CFU/g x 10^5). However, there were substantial variations in the microbial enumeration after the 14 days incubation periods, where most of the bacteria culture plates were too numerous to count (TNC), most especially for experimental treatment pots A, D, E, G and H (please, see key above). However, Fungi isolates gave a very relatively stable colony forming units per gram of sediment (CFU/g) throughout the experimental duration and this was closely followed by Actinomycetes (Table 3). This increased microbial enumeration throughout this study further underscores the fact that microorganisms play very pivotal and significant role in bio remediating heavy metal (Pb) contaminated sediment. These increases in microorganisms after the incubation period may be due to some environmental factors of the sediment such as pH, moisture content, temperature, nutrient etc. This finding is consistent with those of Critter *et al*. (2002), who evaluated both groups of microorganisms in sediment samples quantitatively using agar plate counts and found that amendment with different organic materials affected significantly their quantity (Kubát *et al*., 1999; Zakaria *et al*., 2019; Zahri *et al*., 2020).

Table 4. Concentrations of available Pb in experimental treatment pots before and after incubation (mg/g).

Experimental treatment	Before incubation ($\bar{x}$ ± SD) (mg/g)	After incubation ($\bar{x}$ ± SD) (mg/g)
Soil (A)	4.51 ± 2.38	4.01 ± 0.02
Sediment + Pb (B)	16.39 ± 2.08	15.07 ± 1.87
Sediment + Crude Biosurfactant (C)	3.49 ± 0.09	3.41± 0.35
Sediment Crude Biosurfactant + Pb (D)	15.11 ± 1.77	9.88 ± 0.37
Sediment + Purified Biosurfactant (E)	3.29 ± 0.13	3.26 ± 0.03
Sediment Purified Biosurfactant + Pb (F)	14.64 ± 4.41	9.36 ± 0.22
Sediment + Growth Medium (G)	4.67 ± 0.52	3.05 ± 0.49
Sediment + Growth Medium + Pb (H)	12.95 ± 0.26	10.93 ± 0.84

The colony-forming units of saprotrophic microfungi increased significantly with increasing doses of mineral and organic fertilization; counts of actinomycetes increased in sediments fertilized by mineral fertilizers.

Effect of concentrations of Pb in treatment pots: According to Lloyd *et al.* (2005) microbes are normally employed for the removal of heavy metals from sediment and water. Microbes interact with heavy metals and radionuclides through many identified mechanisms, many of which have formed the basis for potential strategies for bioremediation. Most of the identified mechanism through which microorganisms bio remediate heavy metals includes biosorption (i.e. sorption of heavy metal to cell surface by physiochemical mechanisms), bioleaching (i.e. mobilization of heavy metal via the excretion of organic acids or methylation reactions), biomineralization (i.e. immobilization of heavy metal via the formation of insoluble sulphides or polymeric complexes) intracellular

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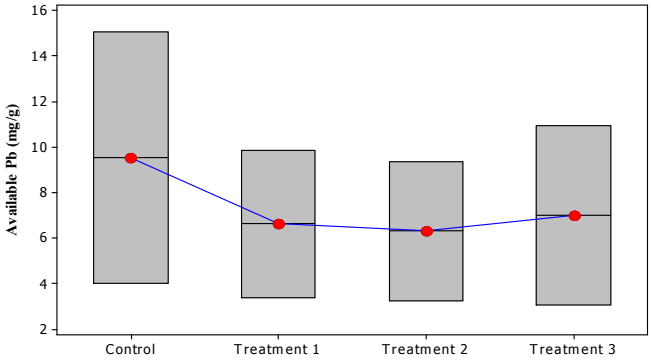
accumulation, and enzyme-catalyzed transformation (i.e. redox reactions) (Dumanovic, 2021). However, biosorption appears to be the most common mechanisms according to Wusheng *et al.* (2018). Figure 2 shows Box-and- Whisker plot indicating the descriptive statistics of available Pb in contaminated sediment after incubation for 14 days. Control medium had the highest available Pb compared to other treatment media. The lowest available Pb was found in (Treatment 2 = Purified Biosurfactant). Figure 3 shows % reduction of available Pb in contaminated sediment after incubation for 14 days. Treatment (F = Sediment + Purified Biosurfactant + Pb) had the highest % reduction of about 35 %, while the lowest was seen in medium (E = Sediment + Purified Biosurfactant) with about 2 %.

Abundance of microorganisms in sediment sample: Figure 2 showed that Treatment 1 (crude biosurfactant) has a mean of 6.65 and a median of 6.65; Treatment 2 (purified biosurfactant) has a mean of 6.31 and a median of 6.31; while Treatment 3 (growth medium) has a mean of 6.99 and a median of 6.65 as against the Control with a mean of 9.54 and a median of 9.54. The control displayed a larger spread followed by Treatment 3 (growth medium) while Treatment 1 (crude biosurfactant) and Treatment 2 (purified biosurfactant) are almost the same although the former (Treatment 1) is larger than the latter (Treatment 2). This shows that purified biosurfactant which was Treatment 2 reduced the amount of available Pb the most with a 36.1 %, followed by crude biosurfactant (Treatment 1) with 34.6 %. Statistically, the *p*-value for the F-statistic is 0.94, confirming that there is no impact of biosurfactant on the remediation of lead (Pb) contaminated sediment. Thus, there is insufficient evidence against Ho. Therefore, the Ho (1) is accepted. It also revealed that there is no statistically significant difference ($p>0.05$) in available Pb concentrations between Crude and Purified Biosurfactants. Thus Ho (2) is accepted (supplementary data). In this report however, Treatment 3 (growth medium) displayed relatively the highest available Pb amongst the three treatments with 15.6 %.

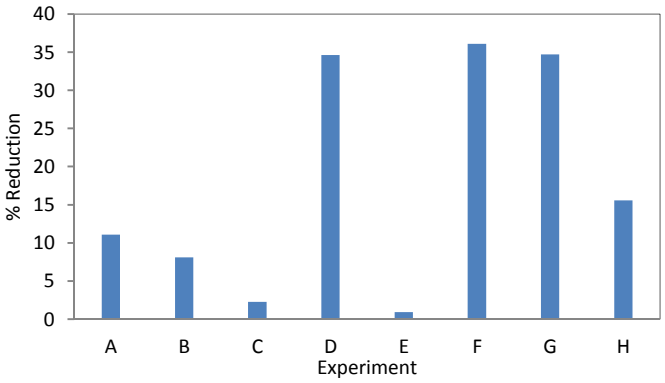
Effect of % reduction of Pb in contaminated sediment: In Figure 3, there is no significant difference in available Pb % reduction between the use of crude and purified biosurfactant with 34.6 % and 36.1 % respectively. From this study, it shows that both crude and purified biosurfactants had meaningful remediation impact on the available Pb contaminated sediment. Till date, there is an increasing interest principally on the use of surfactant to remediate heavy metal contaminated sediment. Studies conducted by (Yan *et al.*, 2020; Saikat *et al.* 2022; Jacob et al., 2018; Awa and Hadibarat 2020) have shown that both biosurfactant and chemical surfactant as bioremediation method, either enhance the chemical removal or degrade of contaminants from sediment.

Fig. 2. Box-and-Whisker plot showing descriptive statistics of available Pb in contaminated sediment after incubation (14 days).



Key:
Control = Sediment
Treatment 1= Crude biosurfactant
Treatment 2 = Purified biosurfactant
Treatment 3 = Growth medium

Fig. 3. % Reduction of available pb in contaminated sediment after incubation (14 days).



Key:
A = Sediment
B = Sediment + Pb
C = Sediment + Crude biosurfactant
D = Sediment + Crude biosurfactant + Pb
E = Sediment + Purified biosurfactant
F = Sediment + Purified biosurfactant + Pb
G= Sediment + Growth medium
H= Sediment + Growth medium + Pb

Conclusion

In this study, the evaluation of the impact of crude and purified biosurfactants synthesized by *Pseudomonas aeruginosa* strain NCIMB 8626 shows no significant difference between crude and purified biosurfactants, hence, determining the potential and most effective forms of the biosurfactant could not be established.

Although, crude and purified biosurfactants seem to have impacted the available Pb present in the sediment after the incubation period of 14 days substantially with 27.41 % and 27.67 % reduction respectively. There is arguably relative increase in microorganisms most especially bacteria in treatment pots containing crude and purified biosurfactants as most were too numerous to count (TNC). However, growth medium containing treatment pots showed a higher enumeration throughout and this may be attributed to other additional nutrient sources provided by the growth medium itself. Generally, all the treatment pots showed significant increase in microbial population after the 14 days incubation periods compared to "before" incubation period.

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