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Remediation of Iron Using Rhamnolipid-Surfactant Produced by *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa was isolated from 20 years abandoned mine site of Itagunmodi Atakunmosa West, Ilesha, Nigeria. Atomic absorption Spectrophotometry (AAS) revealed Fe, Mn, Cr, Zn, Pb and Cr, while, Fe has the highest concentration range of 29-289 ppm in the analysed soil samples. Soil samples were enriched in R2b agar, serially diluted and pour plated. Four bacteria strains were isolated and identified using standard biochemical test. After routine biosurfactant screening by oil spreading and emulsification test, biosurfactant producing bacteria was confirmed as *Pseudomonas aeruginosa*. The partially purified biosurfactants were characterized with TLC and GC-MS analysis. The analyses indicated glycolipid biosurfactant specifically designated as Rhamnolipid-sa1 containing isopalmitic acid, hexadecanoic acid, methyl ester and hydroxylated fatty acid linked to decanoic acids. Iron removal potential of the extracted biosurfactant was studied and the result revealed that Rhamnolipid-sa1 effectively reduced iron (60.34%) and could be useful as alternative remediation tool for treatment of iron contaminated soil.

Key words: Biosurfactant, rhamnolipid, *P. aeruginosa*, iron, bioremediation

INTRODUCTION

Heavy metals are source of serious environmental concern because of their potential reactivity, toxicity and soil mobility. They are released into the environment through various human anthropogenic activities including mining (Galadima and Garba, 2012). Due to ignorance, abandoned mined site potentially rich in heavy metals such as Fe, Zn, Mn, Cr, Pb, Cd and Cu are used for agricultural purpose in some part of the world including Nigeria. These metals are potentially toxic to crops, because they accumulate fast in the vital organ of crops grown in metals contaminated site, thus animal and human that consume such crops are at great danger of heavy metals toxicity (Whang *et al.*, 2008). Iron like cobalt, copper, chromium, manganese and nickel are required in trace amount by plant and human but excessive intake results in a disease condition called Hemochromatosis, inability of the intestine to keep out unneeded iron in the intestine in human and perhaps negatively affect soil microbial diversity (Cremin *et al.*, 2004). Recently, an increase concern about environmental protection has caused the development of cost effective and environmental friendly microbial compounds that exhibit pronounced surface and emulsifying activities called biosurfactant (Morita *et al.*, 2007). Joshi *et al.* (2008) reported biosurfactant as amphiphilic compounds produced on living surfaces, mostly microbial cell surfaces or excreted

extracellularly and contain hydrophobic and hydrophilic moieties. These biosurfactants are classified into low molecular weight such as glycolipid and lipopeptides and high molecular weight bioemulsifier such as lipoproteins, lipopolysaccharides and heteropolysaccharide. Biosurfactants form complexes with metals at the soil interface, followed by desorption of the metal and removal from the soil surface leading to the increase of metal ions concentration and their bioavailability in the soil solution (Mulligan, 2005). Survival of bacteria in heavy metal contaminated soil such as mines could be linked with the utilization of toxic substances such as metal ions by microorganism or microbial products through interactions to concentrate and separate metals by metal binding to the cell surface or within the cell, translocation of the metal into the cell surface and volatilization of the metals as a result of biotransformation reaction and the formation of precipitates by reaction with extracellular polymers or microbial produced anions. The objectives of this study are to isolate and screen biosurfactant producing bacteria, extract and characterize the produced biosurfactant and investigate iron bioremediation potentials of biosurfactant of heavy metal resistance bacteria isolated from mine site.

MATERIALS AND METHODS

Collection of samples: Samples of soil were collected from an abandoned mined site at Itagunmodi, Atakunosa West Local Government Area of Ilesha, Nigeria. The site longitude is 4.7°33'33" N and the latitude is 7.6°16'67" E. The soil samples were collected with the aid of soil auger in sterile polythene bag to the laboratory for microbial, heavy metals and physicochemical analysis.

Bacterial isolation: The media used in this study are R2B Agar and Nutrient Agar. Appropriate quantity of medium was weighed, dissolved in distilled water and sterilized by autoclaving at 121°C for 15 min at 1.06 mmHg (pressure). The sterilized agar medium was allowed to cool to 45°C before pouring to prevent steam formation on the cover of petri dishes. The bacteria were isolated using pour plate technique. The isolated microorganisms were subjected to staining and various standard biochemical tests such as Catalase, Motility, Citrate, Spore, Methyl red, Coagulase, Citrate utilization, Urease, Oxidase, Starch hydrolysis, Indole, Sugar utilization and Gram staining.

Biosurfactant screening, extraction and purification: Oil spreading and emulsification test were used to investigate biosurfactant producing potential of bacterial species isolated from the abandoned mine.

Oil spreading techniques: Twenty micro-litre of crude oil and 20 µL of the supernatant of the culture isolated were carefully added to the centre of petridishes containing 30 mL of water using micropipette. The diameter of the clear zones and the area covered by the oil was measured. This test was done for all isolates separately in triplicate Anandaraj and Thivakaran (2010).

Emulsification assay: The emulsification capacity was evaluated by an emulsification index (E_{24}). Two milliliter of kerosene was added to equal volume of cell free supernatant and homogenized in a vortex at high speed for 2 min. The emulsification stability was measured after 24 h and emulsification index was calculated as below:

$$E_{24} = \frac{\text{Total height of the emulsify layer}}{\text{Total height of the liquid layer}} \times 100$$

The calculation was done for all cultures individually and their emulsification was compared with each other.

Extraction and purification of biosurfactant: The biosurfactant was extracted from cell free-broth at 120 h grown cells via step by step purification of acid precipitates using adsorption chromatography. Bacteria cells were removed from supernatant containing medium by centrifugation at 15,000 rpm for 25 min. The supernatant was subjected to acid precipitation by adding 1 M H_2SO_4 to achieve a final pH of 2.0 and allowing it to precipitate at 4°C. The precipitate was pelleted at 10,000 rpm for 20 min, re-dissolved in distilled water, adjusted to pH 7.0, freeze-dried and weighed. The dried surfactant was extracted with chloroform and methanol with the aid of a rotary evaporator under vacuum (Pruthi and Cameotra, 1997).

Structural characterization of biosurfactant: A spot of crude biosurfactant was placed on the silica plate. The biosurfactant was separated on the plate using chloroform: methanol (10:5). Throne reagent was sprayed to detect glycolipid biosurfactant as yellow spots. The R_f was calculated as per the standard database of biosurfactant (Janek *et al.*, 2010).

The purified supernatant of the extracted biosurfactant was analysed for component identification of the fatty acid present using GC-MS (ALS Vial).

Effect of biosurfactant on iron removal: The extracted biosurfactants was used for removal of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. The nutrient broth containing iron salts was prepared and sterilized. The iron salts were added to the medium at concentration of 50, 100, 150, 200, 250 and 300 ppm per 50 mL of the broth. The pH of the medium was adjusted to 7.0-7.2 and sterilized in the autoclave for 15 min. The extracted biosurfactant (50 μL), was inoculated in the medium and incubated at 30°C for 7 days. The medium with the biosurfactant were kept as treatment and the medium without biosurfactant serve as control. To determine the total metal concentration in the treated medium, the medium was analysed for concentration of heavy metals in Atomic Absorption Spectrophotometer (Bulk Scientific 210VGP).

RESULTS

Soil samples taken for heavy metals analysis were found to contain Cr, Cu, Mn, Zn, Fe and Pb (Table 1). The mean pH and temperature value range from 5.9-6.16 and 23.03-23.20°C, respectively (Table 1). Four different colonies were isolated from agar plates based on the cultural and biochemical test (Table 2). The selected strains were identified as *Staphylococcus aureus* SSI₁, *Pseudomonas aeruginosa* SSI₂, *Escherichia coli* SSI₃ and *Bacillus cereus* SSI₄. Biosurfactant producing ability was found only in SSI₂ indicated by formation of 15 mm oil displacement zone and 45% emulsification activity in oil spreading and emulsification test (Table 3). Other isolate showed very low or negative result Table 3. The TLC analysis showed yellow spot of SSI₂ biosurfactant when sprayed with anthrone reagent with retention factor (R_f) value of 0.3-0.71 (Fig. 1).

The GC-MS analysis of fatty acid detected 16 peaks. Four major peaks selected as parental peaks by GC-MS correspond to long chain poly aliphatic and unsaturated compounds consistent with fatty acid methyl esters linked with decanoic acid are shown in Table 4. The chromatogram of biosurfactant from *Pseudomonas aeruginosa* (SSI₂) is shown in Fig. 2, while the Mass spectra structural results of the chosen fractions are shown in Fig. 3a-d.

Table 1: Physicochemical analysis of the abandoned mined site soil and water samples

Samples	pH	Temp. (°C)	Cr	Cu	Mn	Zn	Fe	Pb
A1	6.46±0.05	23.86±0.05	0.50±0.00 ^a	0.50±0.00 ^b	0.94±0.05 ^a	0.07±0.00 ^a	29.0±1.0 ^a	0.03±0.05 ^a
A2	6.20±0.05	23.16±0.00	0.59±0.01 ^{ab}	0.26±0.00 ^a	1.24±0.05 ^b	0.21±0.00 ^b	131.0±1.0 ^b	0.37±0.50 ^{ab}
A3	5.90±0.05	23.03±0.05	0.77±0.01 ^b	0.51±0.05 ^b	2.30±0.05 ^c	0.55±0.10 ^c	286.0±0.00 ^c	0.52±0.01 ^b

A1, A2, A3 are soil samples, values are Mean±SD. Value followed by similar alphabet along the same column are not significantly different at p = 0.05

Table 2: Morphological and biochemical test of bacteria isolated from soil samples

Lab. ref. number of isolates	SSI ₁	SSI ₂	SSI ₃	SSI ₄
Cultural characteristics				
Colour	White	Green	Creamy	Creamy
Shape	Circular	Circular	Circular	Irregular
Edge	Lobate	Entire	Entire	Rhizoid
Elevation	Raised	Flat	Flat	Flat
Surface	Smooth	Smooth	Rough	Rough
Biochemical test				
Gram's reaction and shape	+ve cocci	-ve Rod	-ve Rod	+ve Rod
Catalase	+	+	+	+
Motility	-	+	+	+
Spore	-	-	-	+
Methyl red	-	-	+	-
Coagulase	+	-	-	-
Citrate utilization	-	+	-	+
Urea	-	+	-	-
Oxidase	-	+	-	-
Starch hydrolysis	+	-	-	+
Indole	-	-	+	-
Sugar utilization				
Glucose	AG	A	A	AG
Lactose	A	-	AG	AG
Sucrose	A	-	-	-
Maltose	A	-	L	L
Mannitol	AG	A	A	-
Probable organism	<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>	<i>E. coli</i>	<i>Bacillus cereus</i>

+ve or +: Positive reaction, A: Acid only, L: Late fermenter, AG: Acid and gas, -ve: Negative reaction, W: Weak reaction, SSI: Soil sample isolate

Table 3: Biosurfactant screening of heavy metals resistant bacteria

Bacteria samples	Oil spreading (mm)	Emulsification (%)
<i>Staphylococcus aureus</i> *	2	-
<i>Pseudomonas aeruginosa</i> **	15	65
<i>Escherichia coli</i> *	-	-
<i>Bacillus cereus</i> *	-	5

*: Negative result, **: Positive result

Table 4: Major compound identified from *P. aeruginosa* biosurfactant by GCMS analysis

RT	Name of compound	Mf	Mw	Quality
18.150	Pentadecanoic acid, 14-methyl, methyl ester (Isopalmitic acid)	C ₁₆ H ₃₂ O ₂	256.42	98
19.957	10- Octadecenoic acid, methyl ester	C ₁₉ H ₃₆ O ₂	296.48	99
23.992	Methyl stearate	C ₁₉ H ₃₈ O ₂	298.50	98
23.895	1, 30 triacontanediol	C ₃₀ H ₆₂ O ₂	454.81	90

RT: Retention time, Mf: Molecular formula, Mw: Molecular weight

Experimental results using Atomic Absorption Spectrophotometer reflected that SSI₂ biosurfactants effectively reduced iron concentration at 50, 100, 150 and 200 ppm, when compared with control. Bioremediation potential of the biosurfactants was more evident at 50 ppm and drastically reduced at 250 and 300 ppm, when compared with the control. The percentage efficiency of the biosurfactant on the removal of iron (Fe) was shown in Table 5.



Fig. 1: Spots of SSI₂ biosurfactant with R_f values of 0.31-0.70

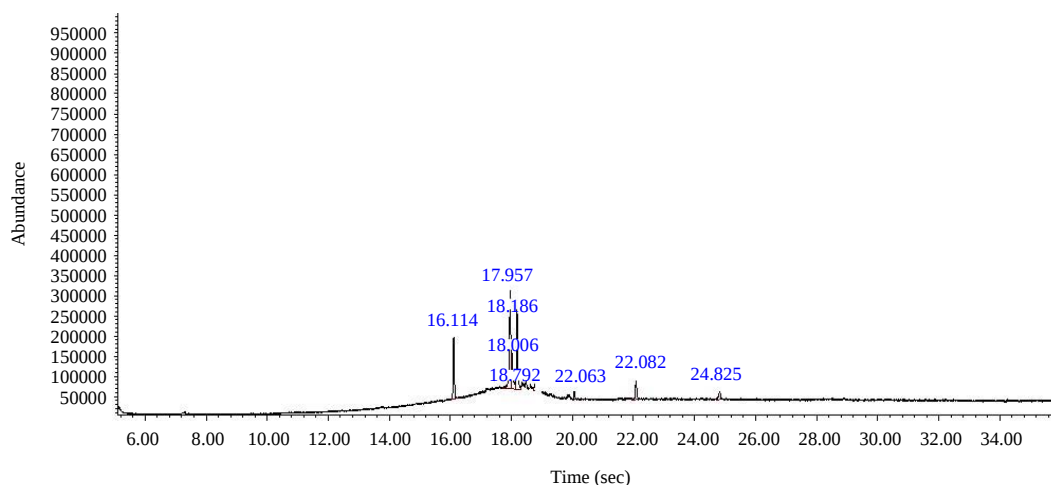


Fig. 2: Chromatogram of *Pseudomonas aeruginosa* biosurfactant SSI₂

DISCUSSION

The values obtained for Copper (Cu), Zinc (Zn) and Manganese (Mn) are not of pressing concern except for the fear of bioaccumulation (WHO., 2011). The obtained concentration for Cu, Zn and Mn are similar to the earlier research done by Matthew *et al.* (2012) who reported that Cu, Zn and Mn concentration in Itakpe mining site are within the WHO recommended permissible limit. The respective concentrations (mean value) of Pb, Cr and Fe range from 0.03-0.93, 0.03-0.77 and 29.0-286 ppm are above maximum permissible limit. Iron has the highest concentration above the estimated toxic level in soil required for agricultural purpose. Although, iron is a trace element but

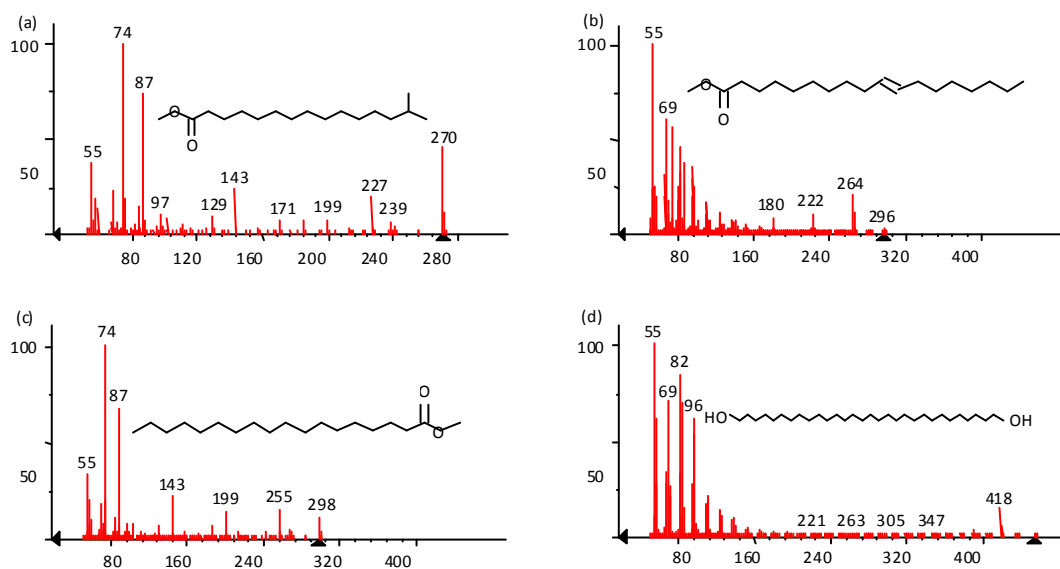


Fig. 3(a-d): GC-MS of biosurfactant sample of *Pseudomonas aeruginosa*, (a) (Replib) pentadecanoic acid, 14-methyl-, methyl ester, (b) (Mainlib) 10-Octadecenoic acid, methyl ester, (c) (Replib) methyl stearate and (d) (Mainlib) 1, 30-Triacontanediol

Table 5: Percentage efficacy of the biosurfactants on the removal of iron (Fe) from the broth media

Strains	Concentration (ppm)					
	50	100	150	200	250	300
SSI ₂	60.34	37.00	28.46	18.00	0.56	0.40
Control	00.00	00.00	00.00	00.00 ^f	0.00	0.00

Values are Mean±Standard Deviation (SD) significantly different at p = 0.05

its excessive intake result in a disease condition called Hemochromatosis, associated with metabolic acidosis, anorexia and Siderosis (Bacon *et al.*, 2011). Oil displacement activities evidenced by the culture supernatants of *Pseudomonas aeruginosa* may be due to the presence of structurally diverse group of surface bio active substances produced by the organisms (Khan *et al.*, 2008). The positive oil displacement and emulsifying activities observed in the organism are in line with the findings of Anandaraj and Thivakaran (2010), who reported zone formation by *Pseudomonas aeruginosa* in a petridish containing 1 mL of coconut oil and gingerly oil. The emulsifying activity of *P. aeruginosa* may be due to its ability to produce extracellular emulsifying agents or discharge of hydrocarbon degrading enzymes during growth of the isolates in the medium. This correlates with the work of researchers such as; Rocha *et al.* (2011) and Parthasarathi and Anna (2014), who reported that variation in emulsification index and stability depend on the rate of production of extracellular emulsifying agents during hydrocarbon breakdown and organisms growth. Chakrabarti (2012) and Thandapani *et al.* (2013) had also used these methods in the isolation of effective biosurfactant producing bacteria, adding that potent biosurfactant production is linked with emulsification efficiency of the organisms. The biosurfactant were confirmed as glycolipid by observing yellow spot on TLC when sprayed with anthrone reagent. The TLC test result confirm to the earlier work done by (Rashedi *et al.*, 2005), who reported rhamnolipid from *Pseudomonas aeruginosa* using anthrone reagent to test for yellow colour spot on TLC plate. R_f values of 0.31-0.71 indicated presence of glycolipid and various rhamnolipid form. R_f value range of 0.19-0.71 has been reported for

mono, di-and various rhamnolipid forms (Donio *et al.*, 2013). The 4 parental peaks chosen by GC-MS in this study have earlier been reported in rhamnolipid produced by *P. aeruginosa* (Elouzi *et al.*, 2012). Lin *et al.* (2012) revealed that the major bioactive fatty acid of *P. aeruginosa* rhamnolipid is Octadecanoic acid linked with alkanoic (acid decanoate) and one or two rhamnose sugar. Spectra analysis of *P. aeruginosa* rhamnolipid have also revealed pentadecanoic acid methyl ester (Isopalmitic acid), stearic acid methyl ester, Heneicosanoic acid methyl ester, Eicosadiene acid methyl ester and heptadecanoic acid methyl ester as fatty acids constituent of Rhamnolipid fatty acid. Hexadecanoic acid, 2-sulfo-1-methyl ester sodium chloride have been reported to have surface active properties which form micelles in water, biodegradable after 28 days and it is useful in detergent formulation (MOE., 2001). Palmitic acid methyl ester was also investigated and reported to have low micelle Critical Micelle Concentration indicating it is probable use as a detergent with less effect on environment (Feigenson, 2006). Vein *et al.* (2008) detailed that 1, 30 triacontanediol promote hydrophobic effect in aqueous system by vesicle formation as a result of biophysical process which may result in formation of micelles, liposome, lipid bilayers as well as form part of the polymorphisms of amphiphile lipid behaviour. The chemical nature of rhamnolipid fatty acids could partly be responsible for its low micelle concentration, foaming nature, biodegradability and environmental friendly nature. Bioremediation potentials of *Pseudomonas aeruginosa* (Rhamnolipid sa1) against the test metal salt of Iron II sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) showed effectiveness at different concentrations. Iron removal efficiency reduced with increased iron concentration. This is in line with the findings of Lidi *et al.* (2012), who reported that the removal efficiency of heavy metals by biosurfactants generally descends with increasing concentration of metals. Heavy metal tolerance and bioremediation potential of *Pseudomonas aeruginosa* have also been reported. Jayabarith *et al.* (2009) reported significant reduction, when *P. aeruginosa* rhamnolipid was inoculated with various concentrations of chromium and zinc. Elouzi *et al.* (2012) revealed that rhamnolipid from *P. aeruginosa* significantly reduced Cd, Pb, Ni, Ba, Zn and Sr by 53, 62, 56, 28, 20 and 7%, respectively at 80 ppm concentration of rhamnolipid. Since biosurfactants are diverse molecule consisting of anionic and cationic structure, they are able to form complex structure with free form of metal residing in solution. The complexation decreases the solution phase activity of the metal resulting in direct contact between the biosurfactant and the sorbed metal, therefore promote desorption (Costa *et al.*, 2010). The potentials exhibited by Rhamnolipids SSI_2 could be due to the presence of various fatty acids in the chemical structure of biosurfactant.

CONCLUSION

On the basis of this study, it was concluded that the *Pseudomonas aeruginosa* was able to produce biosurfactant. Physical and biochemical analysis of the biosurfactant were carried out through standards and TLC techniques. Structure identification and quantification analysis was carried out using GC-MS. The chemical structure of the biosurfactant was identified as glycolipid specifically, Rhamnolipid SSI_2 . The biosurfactant was found to effectively reduced Iron metal signifying that it could be useful as an effective iron bio remediating agent.

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