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RESEARCH ARTICLE

TO EXPLORE ANTIMICROBIAL AND METAL MEDIATION PROPERTIES OF RHAMNOLIPID EXTRACTED FROM *PSEUDOMONAS* SPP. FROM METAL CONTAMINATED SOIL

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Abstract

The heavy metal contamination and disposal of waste frying oil, as well as resistance, occurs due to the hydrophobic nature of antibiotics is a universal issue. The challenge is to develop a method in which biological molecules produced by the low-cost substrate used to reduce metal-associated toxicity in metal-contaminated waste. Along with that, such biological molecules can use alone or synergistically to makes the microbial cell surface more susceptible to accumulate hydrophobic substance (antibiotic) into the cell for restoring resistance. For this purpose, biosurfactant producing bacteria were isolated from a metal-contaminated soil sample and identified as *Pseudomonas spp.*.The result showed that extracellular glycolipid produced by metal isolate was Rhamnolipid. Maximum rhamnolipid production observed when the culture was incubated for 6-8 days in medium containing different amount of mannitol and peptone. Fried oil also acts as the best carbon source giving excellent rhamnolipid production. Moreover, extracted rhamnolipid found to be antimicrobial against gram-positive, gram-negative organisms and fungi as well. It also increases its microbicidal activity of ampicillin antibiotic when used synergistically with rhamnolipid. Different concentrations (60, 80µg/ml) of rhamnolipid were evaluated and compared for the efficiency of heavy metal [Cr (VI)] removal. The result showed that superior adsorbent having 80 µg/ml concentration of rhamnolipid reduced 46%, 39%, 32%, 19% of 10ppm, 20ppm, 60ppm, 80ppm of hexavalent chromium respectively.

Introduction:-

Biosurfactants are naturally occurring amphiphilic surface-active compounds obtain from microbes, which produced either on cell surfaces or secreted extracellularly (Santhini and Parthasarathi, 2014). Rhamnolipids are glycolipids chiefly produced by *Pseudomonas aeruginosa*, and Other *Pseudomonas* species, also known to secrete rhamnolipids, are *Pseudomonas chlororaphis*, *Pseudomonas plantarii*, *Pseudomonas putida*, and *Pseudomonas fluorescens* (Randhawa and Rahman, 2014). Rhamnolipid production was under the control of the quorum-sensing system and occurred after bacterial growth ceased (Das, Yang and Ma, 2014). It is crystalline acid (Randhawa and Rahman, 2014) composed of β-hydroxy fatty acid involves a hydrophilic head containing one or two molecules of rhamnose (Bendaha, M. E, et al.,2012) linked by an α-1,2-glycosidic bond (Nott et al., 20130) to hydrophobic tail containing fatty acids known as monorhamnolipid (rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate) or

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dirhamnolipid (rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate) respectively (Reis, R. S., Pereira, A. G., Neves, B. C., & Freire, D. M. G. , 2011).A heavy metal such as hexavalent chromium is soluble in polluted water and carcinogenic, too (Kim and Om, 2013). The interaction between rhamnolipid and heavy metals leads to the separation of metal from the polluted stream through binding of metal either to the external layer of the microbial cell or within the cell wall barrier that promotes the entry of metal into the cell (Abdurrahim A Elouzi et al., 2012). Extracellular polymers or microbial produced anions facilitate the volatilization of the metal either by the metal precipitation or by biotransformation into the cells (Abdurrahim A Elouzi et al., 2012). Rhamnolipids are known as a source for rhamnose, used for the manufacture of high-quality flavor compounds as well as in cosmetic and healthcare industries, biodegradation and bioremediation of xenobiotics, biocontrol (Vanavil, Perumalsamy and Rao, 2014). Pharmaceutically, antibiotic resistance is a global issue, such as hydrophobic antibiotics are those who do not support entry inside the cells efficiently (K.,A.,L.,T., 2014). Rhamnolipid biosurfactant is known for the uptaking of hydrophobic antibiotics to improve their activity. It also demonstrates antimicrobial activity against various microorganisms by interaction with biological membrane systems, especially phosphatidylethanolamine moiety, which disrupts microbial membranes (Das, Yang and Ma, 2014). This study aimed to check the ability of Rhamnolipids to bind to the heavy metals that usually contaminate water as well as its antimicrobial activity along with comparing the penetration of single antibiotic (ampicillin) and combination with biosurfactant on the target site.

Material and Methods:-

Rhamnolipid production, extraction, and purification:

Metal contaminated soil sample was collected from western railway workshop (Parel) and checked for the presence of *Pseudomonas spp.* as described in Bergey's manual. Biosurfactant producers *Pseudomonas spp.* screened by oil displacement test, drop collapse test, blood agar plate method, and rhamnolipid production confirmed by blue agar plate methodas previously described in Santhini and Parthasarathi, 2014. Rhamnolipid produced (g/L) as described in Hayder, Nadhem,2012 in sterile mineral salt medium (Glucose, 40;NH_4HPO_2, 0.39; Na_2HPO_4, 5.67; KH_2PO_4, 4.08; $FeSO_4.7H_2O$, 0.015; $MnSO_4.H_2O$, 0.002; $MgSO4.7H_2O$, 0.197; $CaCl_2.2H_2O$, 0.001; Distilled water, 1000 ml; pH, 7.) with 2 % olive oil was extracted, purified, and quantified by using orcinol assay (K.,A.,L.,T., 2014). The thin-layer chromatography method performed to characterize the rhamnolipid (Das, Yang and Ma, 2014).

Optimization of conditions for biosurfactant production conditions:

Parameter 1:

Different time interval:

For this, metal isolate grown in 100 ml of Nutrient broth (Himedia) containing 2 % olive oil for 8-10 days (Patil, Pendse and Aruna, 2014) and production of biosurfactant (rhamnolipid) was determined each day by performing oil displacement test.

Parameter 2:

Utilization of substrate:

For this, various isolate had grown in 100 ml of Luria Bertani media (Himedia) with/without olive oil (Abouseoud, Maachi and Amrane, 2007) as well as with 2% waste frying oil (Zhi, L. U. O., Xing, Y., Hua, Z., Guang, Z., Zhi, L. I. U., Ya, Z. H. U., 2013) for 8-10 days under static and shaker conditions. Production of biosurfactant (rhamnolipid) was determined by performing oil displacement test.

Parameter3:

Varying concentration of carbon and nitrogen sources:

3ml of the inoculum (0.1 OD of 18 hours old actively growing culture) inoculated into each of the production medium containing different carbon and nitrogen source (2 % mannitol + 1% peptone and 1 % mannitol + 2 % peptone) as described in Subasioglu,Cansunar(2008) and Rikalovic et al., (2012). The flasks had incubated at 37^0C for 6-8 days under static and shaker conditions and checked for optimum rhamnolipid production by performing orcinol assay, which states rhamnolipid concentration.

Applications:

Heavy metal removal:

For standard chromium sample:

Weighed 141.4 mg of potassium dichromate in 1000 ml of distilled water, which has chromium concentration 50µg/ml (50 ppm). So, to prepare a standard stock [80 µg/ml (80 ppm)] solution, dissolved 226.2 mg of $K_2CR_2O_7$ in

1000 ml round bottom flask and diluted it with distilled water till the mark. Pipette measured volumes from standard chromium solution to give a concentration of 10 ppm, 20 ppm, 60 ppm, 80 ppm. To this, a drop of conc.H_3PO_4 solution and 1 ml of diphenyl carbazide solution added and kept for 5 minutes in the dark to form red-violet coloration and the absorbance measured using a visible spectrophotometer (WPA Biowave II) at 540 nm as described in Kim and Om (2013) and https://www.epa.gov/sites/production/files/2015-12/documents/7196a.pdf.

To determine the highest concentration of rhamnolipid required to remove chromium:
100 ml of chromium (50 ppm or 50μg/ml) solution incubated with 1 ml rhamnolipid having a concentration of 60 μg/ml and 80 μg/ml along with a drop of conc. H_3PO_4 solution, Incubated for 24 hours, then added 1 ml of diphenylcarbazide solution and allowed to stand for 5 minutes in the dark to form red-violet coloration.Absorbance measured using a visible spectrophotometer (WPA Biowave II) at 540 nm.

To determine the removal of chromium (10 ppm, 20 ppm, 60 ppm, 80 ppm) by rhamnolipid:
1 ml 80 μg/ml of rhamnolipidconcentration added into the hexavalent chromium solution of various concentrations (10 ppm, 20 ppm, 60 ppm, 80 ppm) along with a drop of conc.H_3PO_4 solution. Incubated for 24 hours, then 1 ml of diphenyl carbazide solution added and kept for 5 minutes in the dark to form red-violet coloration and measure the absorbance (Elouzi, A. A. et al. 2012).

The percentage of metal removed (η) was calculated based on the original metal content (control) in the aqueous solution using the following equation (Elouzi, A. A. et al. 2012):

η = CM-CMF/ CM × 100 Where; CM- Initial amount of hexavalent chromium metals (control, i.e., without treatment), CMF- Final amount of hexavalent metal chromium (after treatment with Rhamnolipid).

Antimicrobial activity of extracted rhamnolipid:
1 ml culture suspension of pathogenic organisms such as *Candida albicans, Aspergillus niger, Salmonella typhi, Klebsiella pneumonia, Bacillus subtilis, Staphylococcus aureus* inoculated in sterile nutrient agar (Himedia) for bacteria (Govindammal M and Parthasarathi R. 2013) and potato dextrose agar (Himedia) for yeast and fungi (Hayder, Nadhem,2012), and poured in the sterile Petri plate and wells made by using sterile cork borer and filled with extracted crude rhamnolipid as described in Onbasli and Aslim (2008). Then plates incubated at respective temperatures for 24-48 hours and checked for the zone of inhibition.

Combine activity of extracted rhamnolipid with antibiotic ampicillin against Klebsiella pneumonia:
To study the combined effect of extracted rhamnolipid and ampicillin against *Klebsiella pneumoniae* combination of the procedure follows as described in Mizoguchi, Suginaka, and Kotani (1979), White et al., (1996) and Ashayeri-Panah, Feizabadi and Eftekhar, (2014). 1 ml culture suspension of *Klebsiella pneumoniae* of 0.1 OD was mixed with 20 ml of sterile nutrient agar (Himedia) and poured in the sterile petriplate. The sterile filter paper (10 mm) soaked in a crude rhamnolipid solution and other in ampicillin antibiotic solution (1:5 diluted ampicillin injectable 25mg/ml, Sandoz) and placed perpendicular on media to analyze its synergistic action.

Result and Discussion:-

Microorganism and growth:
The *Pseudomonas spp.* obtained from the metal contaminated soil sample. Identification and characterization of the isolate were carried out by biochemical tests, as mentioned in Bergey's manual of systematic bacteriology. The tested isolate showed a positive result for the oil displacement, drop collapse test, and blood agar plateindicates the presence of biosurfactant. The tested strain gives a positive CTAB (Cetyl trimethylammonium bromide) test, which confirms the presence of extracellular anionic glycolipids (rhamnolipids). The result showed that rhamnolipid production was taking place during the stationary phase, where the limitation of a nutrient in the microbial culture promotes rhamnolipid production (Vanavil, B., Perumalsamy, M., & Rao, A. S.,2014). Metal isolate took minimum fivedays for the synthesis of RhlA and RhlB enzymes during the late exponential phase and stationary phase of the growth cycle of different *Pseudomonas* strains (Reis, R. S., Pereira, A. G., Neves, B. C., & Freire, D. M. G., 2011). The produced rhamnolipid was extracted by centrifugation that causes the secretion of rhamnolipid into the broth followed by acid precipitation that neutralizes negative charges present on rhamnolipid, thus becomes less soluble in aqueous broth (Santhini and Parthasarathi, 2014). The existence of a hydrophobic end enables rhamnolipid to solubilize in the organic solvent for extraction (Nordin, N., Zakaria, M., Halmi, M., Ariff, A., Zawawi, R., & Wasoh,

H. 2013). For purification of rhamnolipid, mixtures of solvents used to facilitate adjustment of the polarity between the extraction agent and the rhamnolipid (Santhini and Parthasarathi, 2014). Membrane-associated lipids are polar, which require nonpolar solvent, i.e., methanol, to disrupt hydrogen bonding. Chloroform kills vegetative contamination, and butanol helps to solubilize rhamnolipid. Quantitative estimation of the extracted rhamnolipid had done by using orcinol assay (K. A. L.T., 2014) by calculating rhamnose content. With L-rhamnose, which is part of a rhamnolipid (glycolipid), a standard curve prepared. The extra mass (lipidic part) of rhamnolipid calculated by multiplying rhamnose values by 3.4 coefficient factor (Rahman, P. K. S. M., Pasirayi, G., Auger, V., & Ali, Z.2010); thus, it gives the absolute concentration of rhamnolipid. The thin-layer chromatography result showed only one green spot of monorhamnolipid having R.f value 0.91, which showed related R.f value as standard when compared with standard (Das, P., Yang, X. P., & Ma, L. Z. 2014).

Optimization for rhamnolipid production:

Parameter 1:

Day interval:

The metal isolate showed a maximum production of rhamnolipid in 6-8 days (Graph 1a). It is the time taken by *Pseudomonas spp.* to reach the stationary phase having low nutrient conditions that promote rhamnolipid production (Zhi, L. U. O., Xing, Y., Hua, Z., Guang, Z., Zhi, L. I. U., Ya, Z. H. U., 2013). After the 10^{th} day, there is a decrease in rhamnolipid concentration due to cell lysis caused by its metabolic toxic product formation as well as nutrient depletion, which affectsrhamnolipids production. So, for the optimization, the metal isolate was incubated at $37\ ^{0}$C for 6-8days.

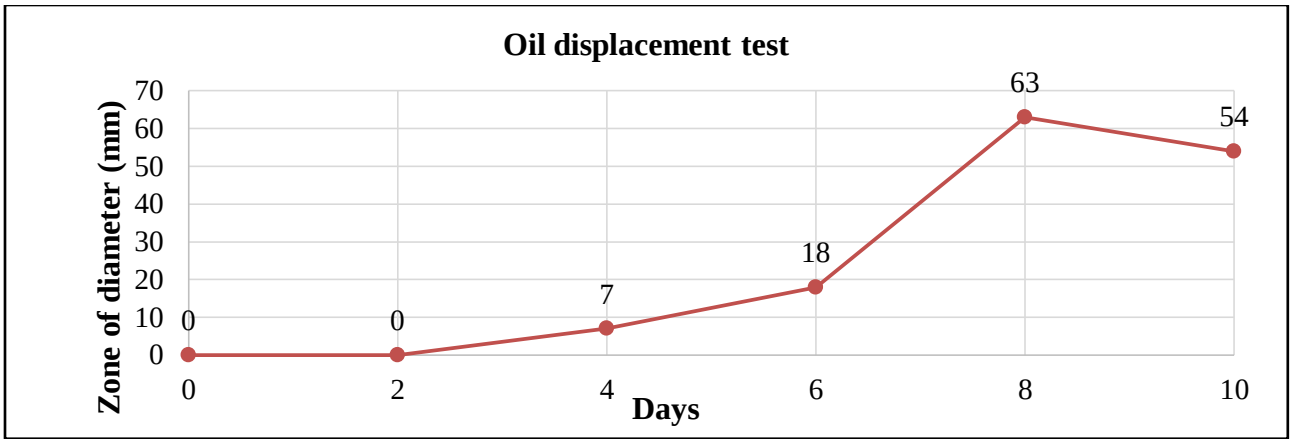
Parameter 2:

Presence of substrate:

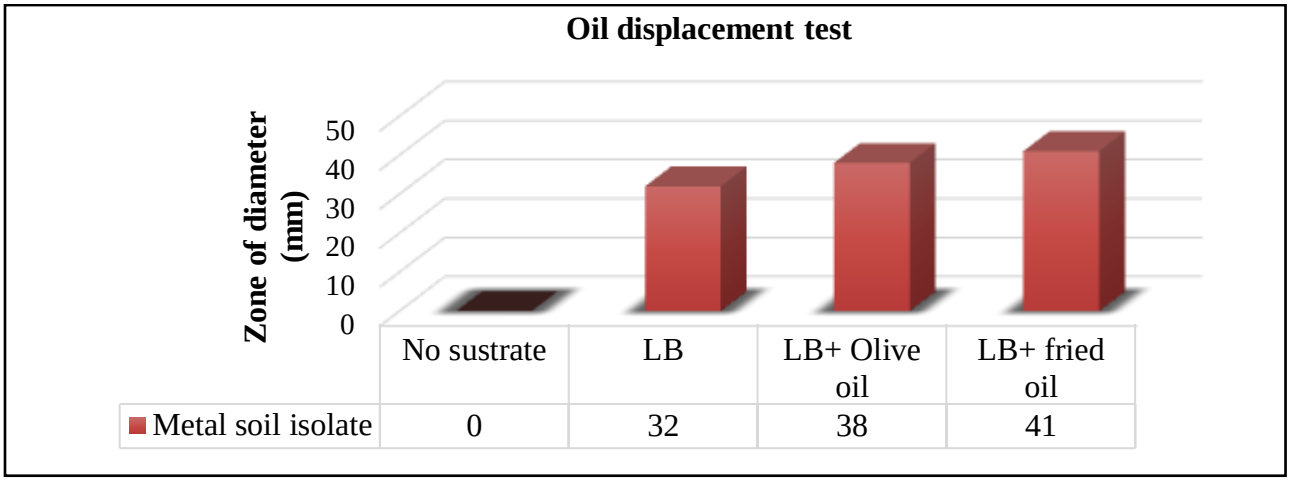
These results (Graph 1b) suggested that higher rhamnolipid production observed under shaker conditions than static conditionsindicate that rhamnolipid production and bacterium growth activity have a close relationship (Zhi, L. U. O., Xing, Y., Hua, Z., Guang, Z., Zhi, L. I. U., Ya, Z. H. U., 2013). The result suggests that culture maintained in Luria Bertani broth also showed rhamnolipid production due to the onset of the stationary phase, and the Limitation of a nutrient in the microbial culture promotes rhamnolipid production. Though this medium rich in nitrogen compounds, supplemented olive oil acts as the best carbon substrate (Abouseoud, Maachi, and Amrane, 2007), which allows for better biosurfactant production; insoluble substrates lead to better performance of rhamnolipids. *Pseudomonas spp.* is lipase positive, which facilitates fatty acid assimilation, which presents in olive oil (Abouseoud, Maachi and Amrane, 2007). Also, less production observed in the absence of olive oil indicates that *Pseudomonas spp.* also produce rhamnolipids when hydrophobic carbon sources were not there. As secondary metabolite, rhamnolipid was usually synthesized under nutrient-limited conditions and act as biosurfactant, which will reduce surface tension between waste frying oil and culture broth, which help microbes to assimilate fried oil containing fatty acids and used as carbon source (Xiangsheng, Z. et al. 2012) leads to limit nitrogen content thus promote rhamnolipid production.

Parameter 3:

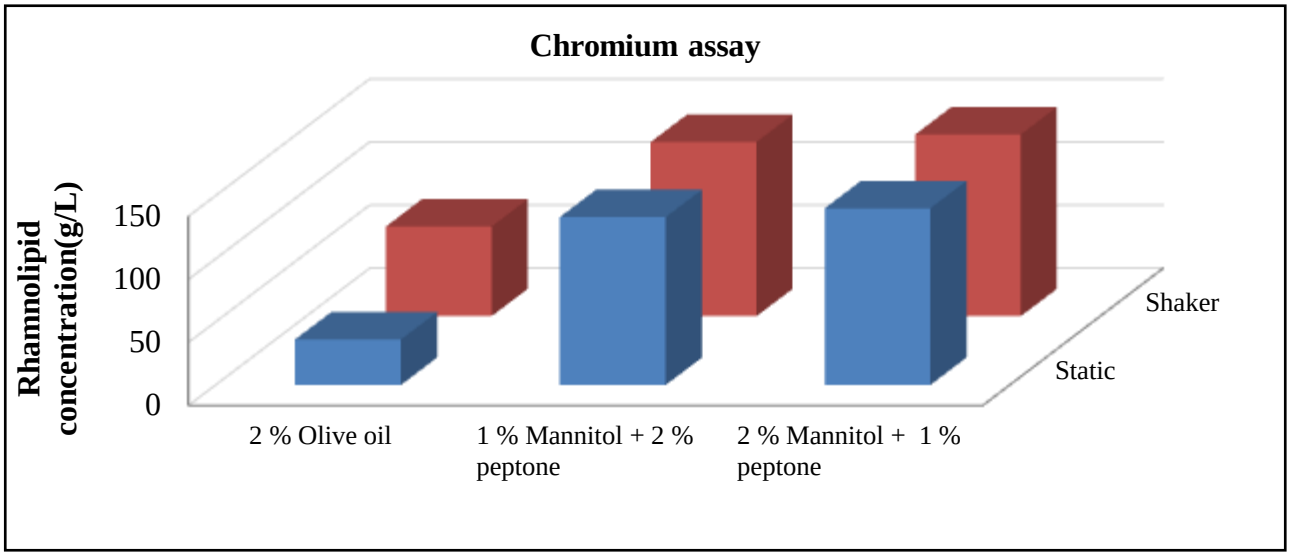
Production of rhamnolipid by *Pseudomonas spp.* in an optimized medium (minimal salt medium incorporated with olive oil, mannitol as carbon source and peptone as nitrogen sources with different concentrations) monitored for 8-10 days (Graph 1c). Rhamnolipid production begins after the 6^{th} day of fermentation (Zhi, L. U. O., Xing, Y., Hua, Z., Guang, Z., Zhi, L. I. U., Ya, Z. H. U., 2013). A significant increase in rhamnolipid concentration observed when culture incubated in medium containing 2 % Mannitol with 1 % peptone, due to the limitation of nitrogen in media favors the product accumulation in the culture broth.With low levels of nitrogen, bacterial growth is limited, which supports the production of secondary metabolites, whereas with excess nitrogen source, the substrate directed towards cellular growth, limiting the accumulation of the product (Rikalovic, M. G., Gojgic, G., Vrvic, M. M., & Karadzic, I. 2012; Subasioglu & Cansunar, 2008).



Graph 1a: Effect of different time interval for rhamnolipid production.



Graph 2a: Efficiency ofrhamnolipid on hexavalent chromium removal.



Graph 1c: Effect of different sources of carbon and nitrogen on rhamnolipid production

Applications:

Heavy metal removal:

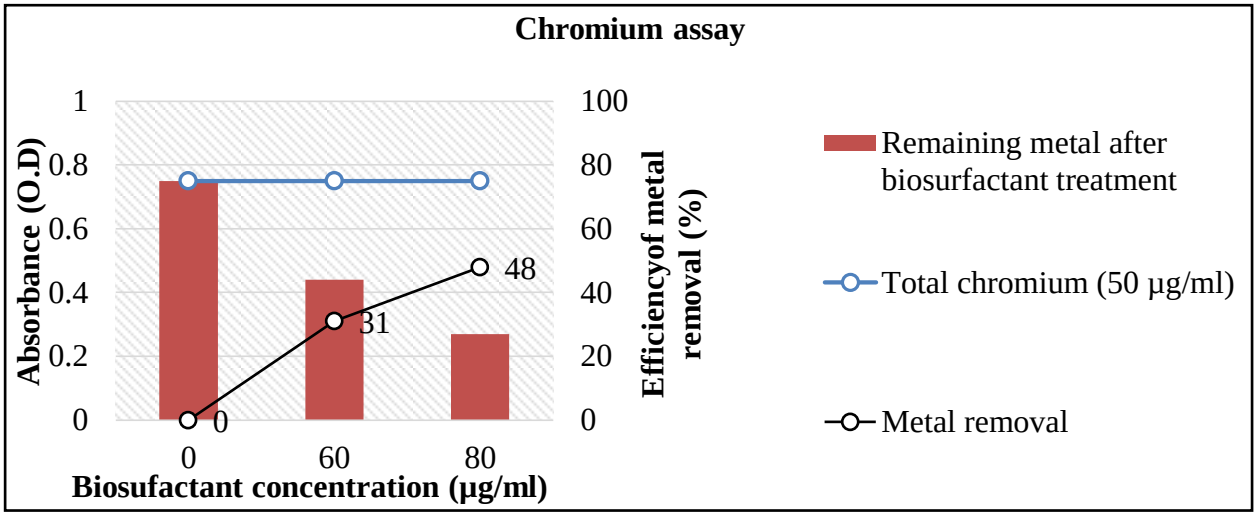
Chromium assay (Method 7196) is used to calculate the concentration of dissolved chromium (VI). Therefore, to determine total chromium, all chromium of potassium permanganate was converted to the hexavalent state by oxidation, forms red-violet coloration when reacted with diphenylecarbazide under acidic conditions. Absorbance measured using a visible spectrophotometer at 540nm (Website - https://www.epa.gov/sites/production/files/2015-12/documents/7196a.pdf.).

To determine the highest concentration of rhamnolipid required to remove hexavalent chromium:

Chromium assay performed to estimate hexavalent chromium concentration in aqueous solution (Table 1). The result showed that 60 µg/ml concentration of biosurfactant removes 31% of chromium (VI), and 80 µg/ml concentration of biosurfactant removes 48% of chromium (VI) and act as a superior adsorbent (Graph 2a). So, 80 µg/ml concentration of biosurfactant used further to check the removal of different concentrations of chromium.

Table 1:- Effect of rhamnolipid on 50 ppm concentration of chromium (VI).

Amount of biosurfactant (µg/ml)	**CM**- The initial amount of chromium (VI) (without treatment)	**CMF**- The final amount of chromium (VI) (After treatment with Rhamnolipid).	**η**-% of metal removal
60	0.75	0.44	31
80	0.75	0.27	48



Graph 2a: Efficiency of rhamnolipid on hexavalent chromium removal.

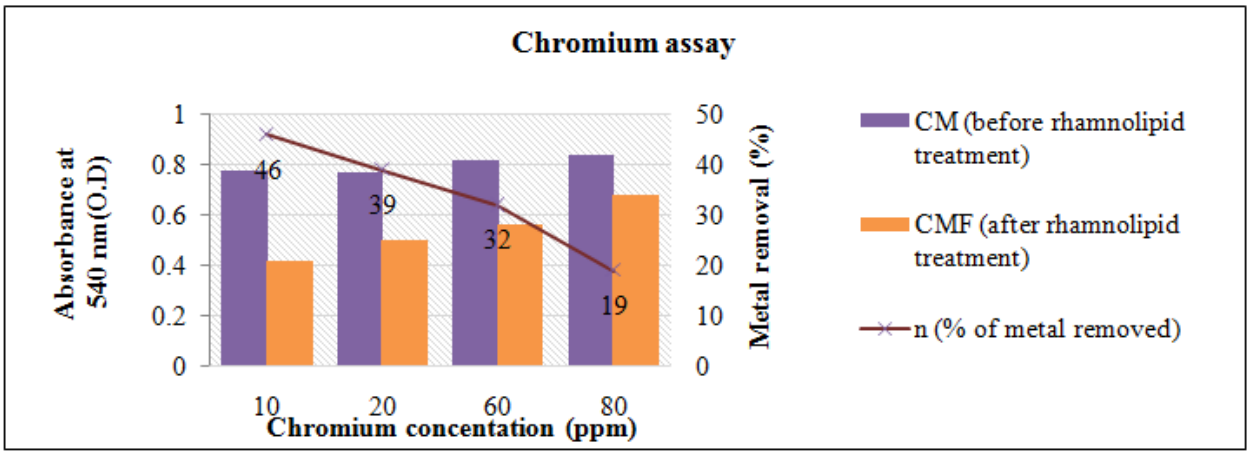
Effect of rhamnolipid on various chromium concentrations:

After the addition of biosurfactant (Rhamnolipid), a remarkable reduction in the chromium(VI) concentration was observed (Table 2). The result showed that 80 µg/ml concentration of biosurfactant removes 46%, 39%, 32%, 19% of 10ppm, 20ppm, 60ppm, 80ppm of hexavalent chromium respectively (Graph 2b). The anionic rhamnolipid carry a negative charge, and thus, when the molecule finds a cationic metal having a positive charge, it forms an ionic bond that is stronger than the bond between the chromium metal and the water, which enables chromium to precipitate (Pacwa-Plociniczak, Plaza, Piotrowska-Seget, & Cameotra, 2011). A higher concentration of metal may affect Rhamnolipid, which ultimately affects metal removal efficiency. The incorporation of biosurfactant (Rhamnolipid) known to bound to the free form of heavy metals from polluted water, which lowers the solution-phase activity of the heavy metal, therefore, promotes desorption (Elouzi, Akasha, Elgerbi, & El Gammudi, 2012).

Table 2: Effect of 80 µg/ml of rhamnolipid on different concentration of chromium (VI).

Amount of chromium (ppm)	**CM**-The initial amount of chromium (VI) (without treatment)	**CMF**-The final amount of chromium (VI) (after treatment with Rhamnolipid).	**η**-% of metal removal

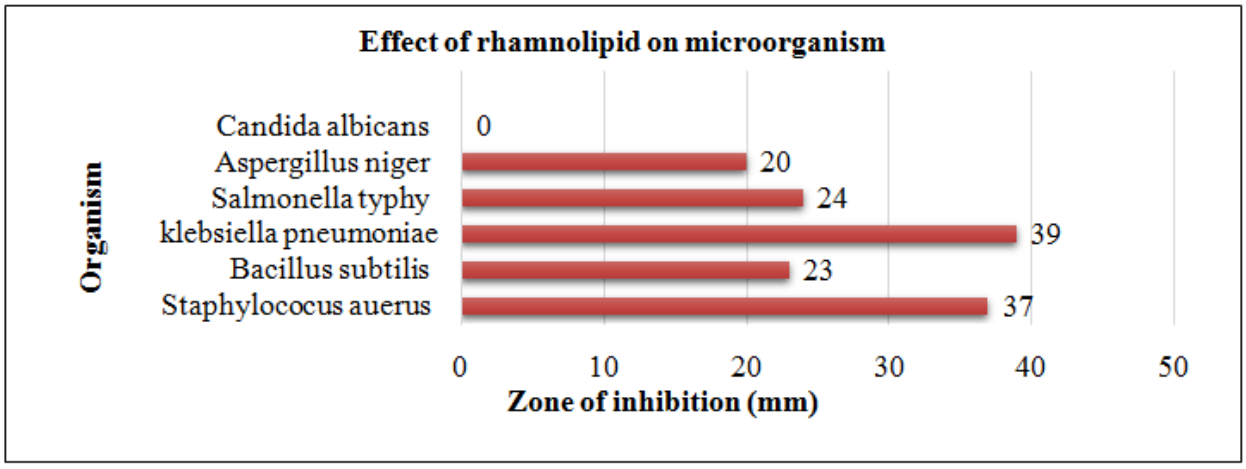
10	0.78	0.42	46
20	0.77	0.5	39
60	0.82	0.56	32
80	0.84	0.68	19



Graph2b:- Chromium metal (VI) removal efficiency of rhamnolipid.

Antimicrobial activity of extracted rhamnolipid:

The result (Graph 3) showed that the antimicrobial activity of extracted rhamnolipid against Gram-positive bacteria were more effective than Gram-negative microorganism. It was achievedby disturbing cell membrane structure by interaction with phospholipids and membranous proteins (Samadiet al.,2012). Also, the lipopolysaccharide content of Gram-negative microorganism membranes limits the entry of rhamnolipid because LPS increases the negative charge of the cell membrane causes repulsive interaction between its lipid portion and anionic rhamnolipid (Samadi et al., 2012). Less zone of inhibition observed in fungi, but yeast has not inhibited by rhamnolipid, because the insufficient concentration of rhamnolipid fails to disturb the cell membrane leads to possess tolerance against rhamnolipid. Metal soil isolates contained only monorhamnolipid, which is known to have a high emulsification index (Das, Yang, & Ma, 2014) that enhances its penetration in the gram-negative cell wall. Adherence of rhamnolipid to the microbial cell wall leads to a breakdown of cell membrane integrity as well as alters its metabolism, which signifies an antimicrobial mode of action (Govindammal M and Parthasarathi R, 2013).



Graph 3:- Antimicrobial activity of extracted rhamnolipid.

Combine action of extracted rhamnolipid and antibiotic ampicillin against *Klebsiella pneumoniae*:

Klebsiella pneumoniae had grown on sterile nutrient agar, on which strips soaked in rhamnolipid and ampicillin solution kept perpendicularly. The unite activity of A & B is larger than that of A alone or B alone (Fig.1) & thus, the inhibition zone is highest at the overlapping zone between A & B indicates a synergistic effect (Table.3). Ampicillin is β -lactam antibiotic bind irreversibly and specifically to the cell wall component, i.e., cell membrane

that inhibits transpeptidase and D-alanine carboxypeptidase activity, which stops cell wall synthesis leads to cell damage (Mizoguchi, Suginaka, & Kotani, 1979). *Klebsiella pneumoniae* has extended-spectrum β-lactamase (ESBL) encoding genes that are mainly present on plasmids that carry antibiotic resistance determinants leads to infection (Ashayeri Panah, Feizabadi, & Eftekhar, 2014). β-lactamase present inside the periplasmic space of the bacterial cell, which hydrolyzes β -lactam ring of ampicillin, thus forms resistance against ampicillin (Mizoguchi, Suginaka, & Kotani, 1979). The cytoplasmic membrane has considered the primary site of cellular damage (Samadi et al., 2012). Ampicillin entry into the cell membrane of β-lactamase producing strains was achieved by rhamnolipid by causing the poresthat increase the cell membrane permeability facilitating the penetration of ampicillin through periplasmic space (Lotfabad, Shahcheraghi, & Shooraj, 2013) results in cell lysis. Therefore, it concluded that the enhancement of susceptibility of *Klebsiella pneumoniae* to ampicillin was probably due to cytoplasmic membrane disruptionand attributed to a significant increase in cell membrane permeability.

A +B = Sum of both effects A and B = Additive Effect.
A + B > Sum of both effects A and B = Synergistic Effect.
A + B < Sum of both effects A and B =Antagonistic Effect.

Table 3:- Combine activity of Extracted rhamnolipid and ampicillin.

No.	Component	Zone of inhibition (mm)
A	Extracted Rhamnolipid	25
B	Ampicillin	27
A+B	Rhamnolipid + Ampicillin	55

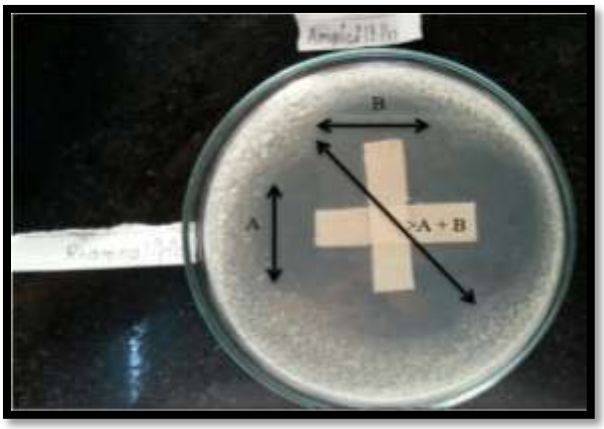


Fig 1:- Combine activity of Extracted rhamnolipid and ampicillin.

Conclusion:-

Our finding has demonstrated that the isolated *Pseudomonas* strain of metal contaminated soil in the western railway workshop, Parel, was found to be the potent producer of biosurfactant (Rhamnolipid). It produces a higher amount of rhamnolipid when waste fried oil supplemented as a substrate. It also possesses antimicrobial activity against pathogenic strain; thus, it may use as an antimicrobial agent after clinical trials. As well as it has the potential to chelate chromium thus can be feasibly utilized towards in situ bioremediation of industrial waste.

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No

Conflict of interest:
No

Funding Sources:
No

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