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Screening and Characterization of Biosurfactant-Producing Bacteria Isolated from Contaminated Soils with Oily Wastes

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Abstract

Biosurfactants are amphiphilic compounds produced by microorganisms and have diverse uses in industry and agriculture. The biosurfactant producing microbes are helpful in bioremediation of heavy metal, pesticides and hydrocarbon contaminated sites. In this study, isolation and identification of biosurfactant producing strain was assessed. Soil samples from soils contaminated with soybean oil refining waste and hydrocarbon were collected. To confirm the ability of isolates to produce biosurfactant, agar blue plate, oil displacement test, drop collapsed assay and measurement of surface tension were conducted. The endogenous isolate ORA9 from contaminated hydrocarbon soil was found to be a good biosurfactant producer. This isolate produced a maximum biosurfactant production of 2,3 ± 0,8 g/L in fried soybean oil. With biochemical and 16s rRNA analysis the isolate ORA9 was identified as *Pseudomonas aeruginosa*. The chromatographic analysis revealed the glycolipidic nature and structural composition of the biosurfactant. The biosurfactant was found to emulsify crude oil and remove heavy metals from contaminated water. These abilities of the isolate can be utilized in environmental bioremediation procedures.

Keywords: Biosurfactants, oily wastes, *Pseudomonas aeruginosa*, heavy metal

1 Introduction

Biosurfactants are amphiphilic surface active agents produced by microorganisms. They are complex molecules comprising a wide variety of chemical structure; their hydrophilic part is usually composed of sugars, amino acids, or polar functional groups like carboxylic acid groups. The hydrophobic part is typically an aliphatic hydrocarbon chain of β-hydroxy fatty acids [1]. These compounds reduce surface and interfacial tensions by accumulating at the interface between two immiscible fluids such as oil and water [2].

In recent years, increasing global environmental awareness has lead to much more interest in biosurfactants compared to their chemical counterparts. It is due to unique properties of biosurfactants including biodegradability, low toxicity, mild production conditions, and environmental acceptability, lower critical micelle concentration, higher selectivity as well as better activity at extreme temperature, pH and salinity [3].

Biosurfactants are produced by diverse groups of microorganisms (i.e., bacteria, yeast and fungus), therefore, the structure and surface properties of these natural products are very different. Biosurfactants are generally categorized into the following groups: glycolipids, lipopeptides, phospholipids, fatty acid salts and polymeric biosurfactants [4]. Biosurfactants have a wide range of industrial applications including, pharmaceutics and medicine, agriculture, food and cosmetic industries [5]. They are also useful in bioremediation of sites contaminated, because they also exhibit natural physiological roles in increasing bioavailability of hydrophobic molecules and can complex with heavy metals, promoting improved degradation of chemical contaminants [6].

The main objective of the present study is to isolate, purify, screen, and characterize endogenous biosurfactant-producing bacteria from contaminated soils from edible oils refining and petroleum refining industries and the evaluation of the structural and physicochemical characteristics and suitability for removing heavy metals from contaminated water will be investigated as well.

2 Materials and Methods

2.1 Microorganism isolation

The soil samples were collected from different localities

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contaminated with soybean oil refining wastes and hydrocarbon. The soil was transferred into sterile flasks glass and stored at 4°C until using. The collected samples were subjected to a sequential enrichment [7] in a mineral basal medium [8]. Soybean oil (1% v/v) was used as only carbon source. At the end of the enrichment culture, samples were serially diluted using sterile saline solution (0.85% NaCl) and different bacterial isolates were selected based on the colony morphology on Tryptone Soya Agar (TSA) plates. The selected isolates were screened for the production of biosurfactants using the following screening methods.

2.2 Qualitative screening of biosurfactant production

2.2.1 Blue agar plate

Isolated strains were screened on blue agar plates, according to the technique described by Siegmund and Wagner [9]. A colony of each isolated was growth in mineral agar medium supplemented with cetyltrimetilammonium bromide (CTAB: 0,5 mg/mL) and metylene blue (0,2 mg/mL). A dark blue halo around the culture was considered as positive for biosurfactant production.

2.2.2. Oil-spreading test

To apply the oil-spreading test, 10 µL of oil was layered over 40 mL of water in a petri plate and a drop of free cell broth was gently added in the centre of the oily layer [10]. The presence of surfactant in the supernatant was detected by the formation of a clear zone due to oil displacement. A water was used as a negative control.

2.2.3. Drop-collapsed assay

The assay was carried out as described by Jain *et al*. [11]. A glass plate was covered with a thin layer of mineral oil and a drop of free cell broth was placed on the hydrocarbon surface. Drop collapse in less than a minute indicated the presence of biosurfactant in the culture medium. A water was used as a negative control.

2.2.4. Hydrocarbon overlay agar method

The hydrocarbon overlay agar method [12] was performed with some modifications. Crude oil-coated nutrient broth agar plates were inoculated with culture of isolates and incubated at 30°C for 48–72 h. A colony surrounded by emulsifed halos was considered positive for biosurfactant production.

Based on the screening test results, the best strain was selected, and then identified by its microscopic appearance and biochemical tests, based on Bergey's manual of determinative bacteriology. Molecular identification by the partial sequencing of the 16S ribosomal gene was done.

2.3 Biochemical and molecular identification of isolated strain.

Biochemical identification of selected isolate include Gram stain, motility test, catalase test, oxidative/fermentative (Hugh-Leifson) test, growth in citrate, pigment production (Agar F, Agar P) and utilization of carbon sources. Genomic DNA was extracted according to the method described by Ausubel *et al* [13]. The primers used for 16S rDNA gene amplification were 27f [14] (5'-AGAGTTTGA TCATGGCTC-3') and 1401R [15] (5'-CGGTGTGTACAAGAAC-3'). Sequence homologies were examined using the BLAST program of the National Center for Biotechnology Information. Multiple sequence alignments were carried out using ClustalW and a consensus neighbor-joining tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software (version 6.06) [16]

2.4 Biosurfactant production

Isolated strains were initially grown in 125 mL Erlenmeyer flask, each contain 25 mL of nutrient broth medium. The flasks were incubated for 24 h at 30°C in a shaker incubator at 150 rpm. Aliquots of 2,5 mL were used to inoculate 25 mL of mineral modified medium [17] with an excess of carbon source (fried soybean oil, 9 g/L approximately) and deficient nitrogen source ($NaNO_3$, 1 g/L). Cultures were incubated for 96 hours in the same conditions of temperature and agitation.

2.5 Isolation and purification of biosurfactant.

After the bacterial cells were removed from the liquid culture by centrifugation (8 000 rpm, 15 min, 4°C), the crude biosurfactant was isolated by adding H_2SO_4 (6 N) to the supernatant. A flocculated precipitate was formed a pH 2 that could be collected by centrifugation (10 000 rpm, 15 min, 4°C). The precipitate was dissolved in a bicarbonate solution (50 mM, pH 7,0) and re-precipitated following the same procedure. The final product was lyophilized.

2.6 Physico-chemical and structural analysis of biosurfactante

2.6.1 Surface tension measurement

Surface tension of the culture broth supernatant was determined by the ring method using a Du Nouy Tensiometer (Krüss, Germany) at room temperature. The tensiometer was calibrated against distilled water. Before each measurement, the ring rinsed several times with distilled water and acetone followed by flaming on Bunsen burner.

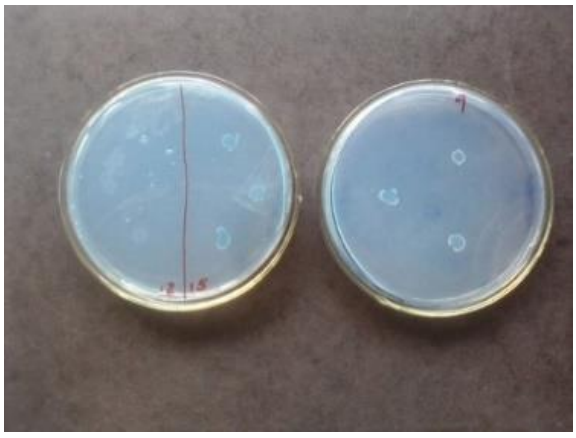

Figure 1: Dark blue colored colonies on blue agar sample volume was 10 µL.

2.6.2 Critical micelle concentration (CMC)

The critical micelle concentration (CMC) of crude

biosurfactant were determined with a Du Nouy Tensiometer (Krüss, Germany) at room temperature. Different concentrations of biosurfactant solution (0–500 mg/L) were prepared by serial dilutions of stock solution in distilled water. CMC was measured from the break point of surface tension versus logarithm of biosurfactant concentration [18]

2.6.3 Emulsification index test

Emulsification index of 500 mg/L aqueous solution of crude biosurfactant was evaluated according to Cooper and Goldenberg method [19], using kerosene and crude oil as hydrophobic phases: 2 mL of biosurfactant aqueous solution was added to 2 mL of hydrophobic compound in a graduated tube. The mixture was then vigorously vortexed for 5 min and allowed to stand for 24 h without any further shaking. After 24 h, emulsification index (E_{24}) was calculated as the ratio of the height of the emulsion layer and the total height of liquid by measuring the emulsion layer formed. A solution of sodium lauryl sulfate 1% and distilled water, were used as positive and negative control, respectively.

2.6.4 Chromatographic methods

To perform a thin-layer chromatography (TLC), sample of crude extract was dissolved in 1mL of chloroform. 100 µL of sample was applied to 20x20 activated silica gel plates G 60 F254 (Merck), and developed in a chloroform:methanol:acetic acid and water (25:15:4:2) (v/v) solvent system. Ninhydrin reagent was used to detect free amino groups. The lipid components were detected in iodine atmosphere. The carbohydrate compound were detected as red spots after spraying the plates with α-napthol in concentrated sulphuric acid.

Gas chromatography (CG) determined the fatty acids composition of the biosurfactant after propanolysis [20]. The propylesters were analyzed in an Agilent 7890A chromatograph equipped with a HP5 capillary column after sample split (1:20). Helium (0.8 mL/min) was used as carrier gas. Injector and FID temperature were 250 and 300ºC, respectively. The oven program was 100ºC by 3 min, increasing temperature at rate of 6 ◦C/min up to 180ºC kept by 5 min. Benzoic acid was used as internal standard. The chromatographic peaks were identified by comparing with the chromatogram of the known fatty acid propyl mixture.

Gradient elution high performance liquid chromatography (HPLC) identified the ramnose sugar after acid hydrolysis with H_2SO_4 10 M solution. The HPLC Ultimate 3000 (Dionex, Sunnyvale, CA, United States) was equipped with HPX-87H column (Bio-Rad, Hércules, CA, United States) with refractive index detector. The flow rate was 0,6 mL/min and the mobile phase used was H_2SO_4 5 mM. The injected

2.7 Application of the biosurfactant in heavy metal removal from water

Biosurfactant suitability for heavy metal extraction was carried out using artificially contaminated water with 500 mg/L of cupper ($CuSO_4 \cdot 5H_2O$), nickel ($NiCl_2 \cdot 6H_2O$) and lead ($Pb(NO_3)_2$) according to method described by Elouzy *et al.* [21]. Aliquots of 10 ml samples (500 mg/L) of the metal solution were transferred into test tubes. From each test tube, 1 mL sample was replaced by either 1 mL of deionized water as control or with 1 mL of rhamnolipid solution (280 mg/L). Each tube was vigorously agitated and then incubated at room temperature for one hour, prior to centrifugation (10 000 rpm, 20 min.) and filtration through Whatman filter paper. The concentration of the heavy metal in the filtrate was measured using inductively coupled plasma optical emission spectrophotometer (ICP –OES).

3. Results and Discussion

3.1 Bacterial isolation

A total of 15 bacterial strains (named as ORA) were isolated including 10 strains from soil contaminated with soybean oil refining wastes and 5 strains from soil contaminated with hydrocarbon. Sixty percent of the bacterial isolates were Gram negative. It has previously been reported that most bacteria isolated from hydrocarbon- or oil-contaminated environments are Gram negative [22][23]. This may be the characteristic that contributes to the majority of the populations surviving such stressful conditions.

In Blue agar and hydrocarbon overlay agar methods, of the 15 isolates, only 33% produced dark blue halos and emulsified halos around the colony, respectively, and were considered as positive for biosurfactant production (Fig. 1)

Except ORA9 strain, isolated from hydrocarbon contaminated soil, the other fourth isolates did not show biosurfactant production in the remaining two screening methods: drop collapsed and oil spreading tests. Similar result was reported by Burch *et a*l. [24], in isolating bacteria that exhibited preferential production of biosurfactants on agar plates, compared to cultures in liquid medium, which could be due to a stimulatory effect by increasing the viscosity of culture medium.

Table 1: Identification and characterization of ORA9 strain

Test	Result
Gram	-
Catalase	+
Oxidase	+
Motility	+
Sugars fermentation:	
Glucose	+
Maltose	-
Lactose	-
Test O/F	O
Agar F	Green pigments
Agar P	-
Citrate	+

This result confirms the point made by Sapute *et al*. [12] suggesting that a single method is not suitable to identify all type of biosurfactant. Therefore, a combination of various methods is required for effective screening.

3.2 Identification of selected strain

Identification of selected bacterial isolate was done using morphological and biochemical methods based on Bergey's manual of determinative bacteriology. Results of various biochemical tests and general characteristics of selected isolate are presented in Table 1. The bacterium was

gram-negative, motile rod-like, and grew aerobically. This strain formed entire smooth, convex, and wet colonies, which was slightly circular with diameter 1–2 mm within 1–2 days. The
colony could easily scrape off from nutrient agar plates incubated at 30°C.

Partial 16S rRNA sequence alignment revealed that ORA9 strain was closely related to the species in genus *Pseudomonas*. ORA9 strain also exhibited the highest similarity (99%) to *P. aeruginosa* (Fig. 2). We thus tentatively classified ORA9 strain as *P. aeruginosa* ORA9. The partial 16S rRNA gene sequence of the selected isolate is available in the Genbank under accession number of KJ645898.

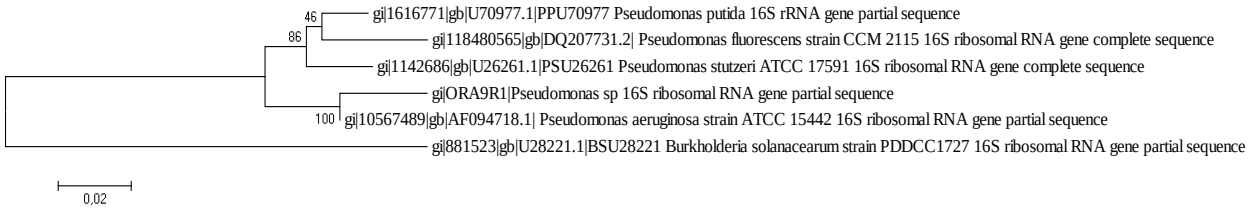

Figure 2: Phylogenetic tree obtained by neighbor-joining analysis of 16S rRNA gene sequences, showing the position of the biosurfactant producing ORA9 strain among neighboring species of the genus *Pseudomonas*

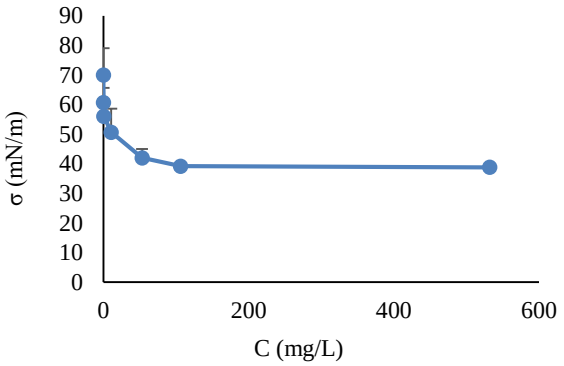

Figure 3: Surface tension versus concentration of crude biosurfactant ORA9

3.3 Biosurfactant production

Biosurfactants can only be considered as substitutes of synthetic surfactants if the cost of the raw material and the process is minimal. The use of alternative substrates such as oily wastes is one of the attractive strategies for economical biosurfactants production [25]. On the other hand, the world production of vegetable oils is about 2,5-3 million tons/years, 75 % of which are derived from plants. Most of these oils are used in the food industry, especially for frying processes, and generate great quantities of by-products and wastes [26]. Therefore, fried soybean oil was applied as the carbon source for biosurfactant production in this research. *P. aeruginosa* ORA9 strain produced 2,3 ± 0,8 g/L of biosurfactant when grew in mineral medium with fried soybean oil as only carbon source.

Waste frying oil has great potential for microbial growth and transformation: of the thirty-six strains screened, nine *Pseudomonas* showed satisfactory growth and surface activity properties [27]. Makkar *et al*. [28] reported that residual cooking and/or waste frying oils are a major source of nutrient rich low cost fermentative waste. Ali Raza *et al*. [29] reported a feed batch process with waste soybean frying oil for rhamnolipids production (9,3 g/L) with a production yield of 2,7 g/L. *Pseudomonas aeruginosa* LBI produced 11.7 g/L of rhamnolipids when grown in soybean soapstock waste [30]. A maximum yield of 7.6 g/L of biosurfactant was obtained when *Pseudomona*s sp grown on used edible oil as a carbon source [31]. Ali Diab *et al* [32] reported an estimated of 2.8 g/L of biosurfactant produced by *P. aeruginosa* SH 29 when grown in waste frying oil (2% w/v).

3.4 Physico-chemical characterization of biosurfactant

3.4.1 CMC and surface tension

One of the most important properties of a surfactant is their spontaneous aggregation in water and formation of well-known structures such as spherical micelles, cylinders, bilayers, etc. The surface tension decreases gradually with increasing surfactant concentration. At a certain concentration called critical micelle concentration (CMC), this decrease stops. Above the CMC, the surface tension remains almost constant [33]. Fig. 3 shows the plotting of surface tension versus logarithmic concentration (mg/L) of biosurfactant produced by ORA9 strain. The surface tension of distilled water decreased gradually, with increasing biosurfactant concentration, from 72 mN/m to 39 mN/m, with CMC values of 87,47 mg/L. This CMC was much lower compared with some synthetic surfactants. For instance, sodium dodecyl sulfate (SDS) has a CMC value of 2,56 g/L [34].

CMC showed that surfactant is efficient, i.e., less surfactant was necessary to decrease the surface tension [19]. CMC values at a wide range from 5 to 386 mg/L have been reported for biosurfactants [1] [35]. Abalos *et al*. [18] reported CMC values of 106, 150 and 234 mg/L for different mixtures of mono and di-rhamnolipids produced using soybean oil refinery wastes. Other CMC values reported for biosurfactants produced by *Pseudomonas* strains are 26.5 mg/L [36], 50 mg/L [37], 28.8 mg/L [38], 115 mg/L y 130 mg/L [39]. Molecular structural differences including ratio and composition of homologues, the presence of unsaturated bonds, the branching and length of aliphatic chains of rhamnolipids can explain different CMC values of biosurfactants produced by *Pseudomonas* strains [40]

3.4.2 Emulsification index determination

The E_{24} (%) of aqueous solution of biosurfactant produced by ORA9 strain was examined with kerosene and crude oil. The ORA9 biosurfactant was less efficient to emulsify kerosene (11 %) compared to crude oil (55 %), showing potentiality to be used to facilitate the availability of this contaminant in environmental treatments. For the detection of potential strain, Femi-Ola *et al*. [41] measured the E_{24} value of isolates and showed 53,13 % emulsification activity with kerosene. Ann Joice and Parthasarathi [42] used different hydrocarbon and vegetable oil for emulsification activity. They found biosurfactant isolated from *P. aeruginosa* PBSC1 showed maximum emulsification activity against crude oil. Velmurugan *et al*. [43] reported emulsification index of 44% against kerosene of biosurfactant produced by the isolate H11.

3.5 Structural analysis of biosurfactant

The crude extract of biosurfactant was analyzed by analytical TLC. Essentially, one spot with Rf value of 0.73 was observed. The presence of carbohydrates induces less hydrophobicity to the compound and hence the monorhamnolipid had greater Rf values when compared to the dirhamnolipid [44] The spots showed a positive reaction in iodine atmosphere and with α-napthol, indicating its glycolipid nature.

The glycolipidic biosurfactants produced by *P. aeruginosa* is a heterogeneous mixture of rhamnolipids homologues of mono-rhamnosidyl (R1), or di-rhamnosidyl (R2) residues, with a variety of alkyl-chains, which depends on the composition of the substrate supply in the culture. Nevertheless, the main homologues produced (regardless of the substrate or strain) are L-rhanmosyl-β-hydroxydecanoyl-β-hydroxydecanoate (R1C10C10) and L-rhanmosyl L-rhanmosyl-β-hydroxydecanoyl-β-hydroxydecanoate (R2C10C10). However, most of the biosurfactants produced by *Pseudomonas aeruginosa* strains are dirhamnolipids. The difference in types and proportion of rhamnolipids in the mixture might result from the age of the culture, bacterial strains, specific culture conditions and substrate composition [45].

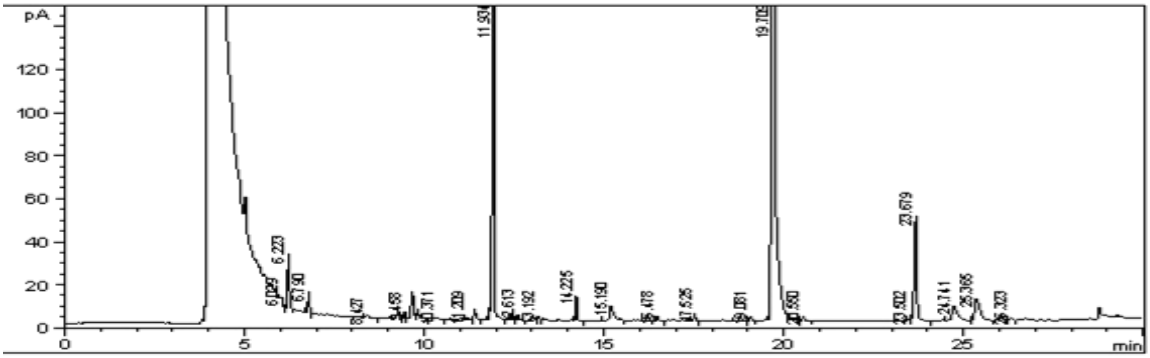

Figure 4: GC chromatogram of rhamnolipids produced by ORA9 strain for fried soybean oil.

Table 2: The effect of ORA9 biosurfactant on heavy metals removal from aqueous solution

Metal	Control (mg/L)	Samples after treatment with biosurfactant (mg/L)	Removal efficiency (%)
Pb^{2+}	432,37 ± 9,84	178,34 ± 26,90	59
Cu^{2+}	448,79± 20,91	168,33 ± 2,57	62
Ni^{2+}	644,65 ± 5,43	260,72 ± 12,22	60

In this study only one spot in TLC was obtained suggesting prevalence of monorhamnolipids homologues in the mixture excreted by *P. aeruginosa* ORA9. The structural characterization by HPLC confirmed the presence of rhamnose sugar and GC analysis showed 3-hidroxydecanoic acid (C10:0) as the main constituent with more than 90 mol% in excreted product. Other fatty acid was detected but in low amounts, that is 3-hidroxydodecanoate (C12:0), with prevalence of 5 to 7 mol%, and 3-hidroxioctanoate (C8:0) with approximately 3 mol% (Fig. 4).

The composition of rhamnolipids mixtures produced is important, since this defines the physicochemical properties of the products, which further impacts on their potential application [46].

Rikalović *et al*. [47] reported the characterization by electrospray ionization mass espectrometry (ESI–MS) of rhamnolipids produced by *Pseudomonas aeruginosa* san-ai strain, confirming the presence of homologues R1C8C10/R1C8C10, R1C8C12/R1C12C8//R1C10C10, R1C10C12//R1C12C10, R2C10C12:1/R2C12:1C10, R2C10C10, in the mixture.

Abassi *et al*. [3] reported the composition of a biosurfactant excreted by *Pseudomonas aeruginosa* MA01 strain cultivated in soybean oil. The analysis indicated the presence of three major monorhamnolipid species: R1C10C10, R1C10C12:1, and R1C10C12; as well as another three major dirhamnolipid species: R2C10C10, R2C10C12:1, and R2C10C12.

George and Jayachandran [48] reported the presence of one dirhamnolipid R2C10C10 and two monorhamnolipid forms R1C12C10 and R1C10C8/R1C8C10 as major components, in the characterization of rhamnolipid biosurfactant from waste frying coconut oil using a novel *Pseudomonas aeruginosa* D.

3.6 Heavy metal removal

Metals and metalloids such as mercury, cadmium, lead, chromium, copper and nickel can threaten ecosystems and human health through either the food chain or direct exposure to contaminated soil and water. Unlike organic contaminants, heavy metals are not biodegradable and tend to accumulate in living organisms, and are known to be toxic or carcinogenic [49].

Table 2 show the results obtained on the removal of heavy metals from aqueous solution by ORA9 biosurfactant. Compared to control, a significant effect in reducing the heavy metals concentration after the addition of biosurfactant was observed. The removal efficiencies achieved was similar for the three metals, indicating similar affinity to form complex with a biosurfactant.

Elouzi *et al*. [21] reported removal efficiencies of 62% and 58% for lead and nickel, respectively, with 80 ppm of biosurfactant. Pathaka and Nakhate [50] reported a reduction of heavy metal concentration to 71% for $Pb(NO_3)$ when applied rhamnolipid produced by *Pseudomonas aeruginosa* MTCC 1688 in contaminated water.

The usefulness of biosurfactants for remediation of heavy metal contaminated water is mainly based on their ability to form complexes with metals. The anionic biosurfactants create complexes with metals in a nonionic form by ionic bonds [51]. This decreases the solution-phase activity of the metal resulting in direct contact between the biosurfactant and the sorbed metal and, therefore, promotes remotion. (Elouzi 2012).

4 Conclusions

In the present study, 15 bacterial cultures were isolated from hydrocarbon and soybean oil refining waste contaminated soil. Among them, only one strain was capable to produce biosurfactant in mineral medium with soybean-fried oil as carbon source. The biosurfactant producer Pseudomonas aeruginosa ORA9 gave maximum 2,3 ± 0,8 g/L biosurfactant production. The biosurfactant exhibited high emulsification ability with crude oil and adecuated desorption efficiencies on removing heavy metals from contaminated water. These properties suggest its potential application in environment clean up procedures.

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