IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 12, Issue 6 Ver. I (Nov. – Dec. 2017), PP 13-18
www.iosrjournals.org

Characterization of Biosurfactant Produced by *Pseudomonas putida* PS6 Isolated from Contaminated Soil

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Abstract: *Biosurfactants are surface active compounds that reduce the interfacial tension between two liquids or between a liquid and solid, these compounds produce extracellularly or as part of the cell membrane in bacteria, yeast and fungi. In this study the Pseudomonas putida PS6 isolated from soil contaminated with hydrocarbons and it showed the highest capability to produce the biosurfactant when grown in BH media with 1% of diesel oil as carbon source. The biosurfactant was extracted from cell-free supernatant of Pseudomonas putida PS6 culture by chloroform: methanol (2:1) and then identified based on the methods of TLC, chemical compositions, blue agar plate and FTIR analysis. The results from all methods indicate that the biosurfactant can designated as glycolipid. The biosurfactant showed emulsification activity on a wide range of temperature between (20-100˚C) and gave the highest emulsification index 74.6% at pH 7, while the stability of biosurfactant decreased at acidic and alkaline conditions, also in the study the effect of salinity, the produced biosurfactant was stable in different concentration of NaCl between 5-15%. This biosurfactant showed high stability under studied conditions, therefore can be used in industrial applications.*

Key words: *Pseudomonas putida, biosurfactant, glycolipid*

Date of Submission: 29-09-2017 Date of acceptance: 07-11-2017

I. Introduction

Soil pollution with petroleum products has become one of the main concerns due to its content of chemical and hazardous materials, which alerts the whole environment, and finding solutions to overcome this problem would be very important. Many techniques have been discovered and examined for soil treatment and one of the most applicable methods is soil washing by surfactants. Among the soil washing methods, biosurfactants use is promising because of its efficiency for remediation of oil-contaminated soils and less environmental impacts from residue compared to surfactants [1].

Biosurfactant are amphiphilic biological compounds produced extracellular or as part of the cell membrane by a variety of yeast, bacteria and filamentous fungi [2].Biosurfactants are amphiphilic, consisting of two parts, a polar (hydrophilic) moiety and a non-polar (hydrophobic) group. The hydrophilic group consists of mono-, oligo-, or polysaccharides, peptides or proteins while the hydrophobic moiety usually contains saturated, unsaturated and hydroxylated fatty acids or fatty alcohols [3].

Due to their amphiphilic structure, biosurfactants increase the surface area of hydrophobic water-insoluble substances, increase the water bioavailability of such substances and change the properties of the bacterial cell surface. Surface activity makes surfactants excellent emulsifiers, foaming and dispersing agents [4].

In comparison with synthetic surfactants, biosurfactants have better surface activity, lower toxicity, they can bind heavy metals, have higher biodegradability, selectivity and biological activity, they are produced from renewable resources, can be produced through fermentation and can be reused by regeneration [5, 6]. They are active at extreme temperatures, pH and salinity as well, and can be produced from industrial wastes and from by-products. This last feature makes cheap production of biosurfactants possible and allows utilizing waste substrates and reducing their polluting effect at the same time[8,9].

These properties have allowed use of biosurfactants in the remediation of inorganic compounds such as heavy metals [8, 10] and in the remediation of organic compounds such as hydrocarbons [11]. Also, the ability to reduce the interfacial tension of oil in water has allowed applied of biosurfactants for the removal of water from emulsions prior to processing [12] and therefore they are applied in oil recovery [13,14]. Because of their potential advantages, biosurfactants are widely used in paint, cosmetics, textile, detergent, agrochemical, food and pharmaceutical industries [15,16].

Biosurfactants are categorized by their chemical composition, molecular weight, physico-chemical properties and mode of action and microbial origin. Based on molecular weight they are divided into low-molecular-mass biosurfactants including glycolipids, phospholipids and lipopeptides and into high-molecular-mass biosurfactants/bioemulsifiers containing amphipathic polysaccharides, proteins, lipopolysaccharides,

lipoproteins or complex mixtures of these biopolymers. Low-molecular-mass biosurfactants are efficient in lowering surface and interfacial tensions, whereas high-molecular-mass biosurfactants are more effective at stabilizing oil-in-water emulsions [17,18].

The most commonly isolated biosurfactants are glycolipids and lipopeptides. In oil fields, some but not all effective oil-degrading bacteria produce extracellular biosurfactants to facilitate microbial oil uptake and degradation by emulsifying the hydrocarbon [19].Surfactants and biosurfactants can increase the pseudo-solubility of petroleum components in water [5]. The objectives of this study were to produce biosurfactant by *P.putida* and study its stability in extreme environmental conditions. The strain producing biosurfactant can be suitably used in oil field, biomedical and environmental applications.

II. Material and methods

2.1 Bacterial isolate

The isolate *pseudomonas putida* PS6 used in this study was isolated from soil sample contaminated with hydrocarbons and identified depending on morphology, biochemical tests and Viteck2 system. This step done in a previous research.

2.2. Production medium and cultivation conditions

The Bushnell Hass medium described by [19], with the following composition (g/l)); $NH_4NO_3$1.0, $FeCl_3$ 0.05, KH_2PO_4 1.0, K_2HPO_4 1.0, $MgSO_4$ 0.2, $CaCl_2$ 0.02 was used as production and cultivation medium, the pH was adjusted to 7.0 by using 1N NaOH and the diesel oil 1% was added as carbon source. The flasks were inoculated with isolate and incubated in shaker incubator (150 rpm) at 35°C for 10 days.

2.3. Extraction of biosurfactant

The cell free supernatant was adjusted to pH 2 by HCl (6N)and allowed to stand overnight at 4ºC , then extracted with equal volume of chloroform : methanol mixture (2:1 v/v). The aqueous layer at the top of the separation funnel was removed and the emulsifier layer was collected, then the solvent was evaporated[20].

2.4. Analysis of biosurfactant

2.4.1. TLC analysis

0.04 g from extracted biosurfactant was dissolved in 1 ml of chloroform: methanol mixture, then 10 µl from them was spotted on pre-coated silica gel (20 ×20)cm . A mixture of chloroform: methanol: acetic acid (65:15:2, v/v/v) was used as a separating agent [21]. Detection of the main components were achieved by exposing the plates of silica gel to iodine vapor, ninhydrin reagent, phenol reagent and orcinol reagent to detect the lipids, amino acids, sugars, and glycolipids group, respectively. The reagents were sprayed followed by heating at 110 ˚C until the detection of the definite spots [22].

2.4.2. Chemical contents in biosurfactant

The protein content in the biosurfactant was estimated by using lowry method [23]. While the total carbohydrate content was estimated using the phenol-sulfuric acid method [24]. Whilst the lipid content was estimated based on the method described by[25].

2.4.3 Detection of glycolipid biosurfactant

Production of glycolipid biosurfactant in the studied bacterial isolate was detected using the blue agar assay. The blue agar plates were prepared from mineral salts agar medium supplemented with 0.2 mg/ml of Cetyl trimethylammonium bromide and 0.005mg/ml of methylene blue.50 µl from cell-free supernatant containing the biosurfactant was pipetted into wells made in blue agar plates, the plates were incubated at 37 °C for 24–48 h. After incubation period the appearance of blue halo around the wells suggests the glycolipid nature of this biosurfactant[26].

2.4.4 Detection by Fourier Transform Infrared Spectroscopy

The biosurfactant was analyzed by FTIR spectroscopy method by mixed the dried biosurfactant with potassium bromide (KBr) crystals at an approximate ratio of 1:10 (w/w) using mortar and pestle, then placed in cap and compressed to form a thin pellet. The spectrum of the pellet was obtained by a Shimadzu FTIR spectrophotometer.

2.5 Stability of biosurfactant

The effect of temperature, pH and NaCl concentration on the stability of biosurfactant were studied using 3 mg of dry biosurfactant dissolve in 1ml of Tris-HCl(0.1M). The solutions of biosurfactant were incubated for 30 min at different temperatures (20-100ºC) to study the thermal stability, while the effect of pHs

values were investigated in different solutions adjusted by using HCl /NaOH (1N) to obtain the pHs values between 2-11. Whilst the different concentrations of NaCl were prepared to obtain the final concentrations 5-20% (w/v) with control. The activity of biosurfactant was determined according to E24% [27].

III. Results and Discussion

3.1 Analysis of biosurfactant

3.1.1Thin layer chromatography TLC

The components of the biosurfactant producing from *pseudomonas putida* were appeared as single spots on TLC plates, with retention factor (Rf) values of 0.75, 0.18, 0.64 and 0.66 for lipids, amino acids, carbohydrates and glycolipid respectively. These spots gave positive reactions for lipids , carbohydrates, glycolipid and amino acid when expose to iodine vapor, phenol reagent, orcinol-sulfuric acid reagent and ninhydrin reagent respectively.

3.1.2 Chemical contents in biosurfactant

The results in table (1) revealed that the 1 mg of biosurfactant extracted from *pseudomonas putida* PS6 contains 0.3606 mg of carbohydrates, 0.60 mg of lipids and 0.033 mg of protein, therefore this biosurfactant consist from 36.29% carbohydrates, 60.38% lipids and only 3.32% protein.

Table (1): Concentrations of carbohydrates, lipids and protein in biosurfactant producing from *pseudomonas putida* PS6

Components	Concentration (mg) in 1 mg biosurfactant	Percentage of component (%)
Carbohydrates	0.3606	36.29
Lipids	0.60	60.38
Protein	0.033	3.32

3.1.3 Methylene blue agar test

The results from method of blue agar plates were appeared a dark blue halo around the wells of biosurfactant as an indicator for presence of anionic biosurfactant such as glycolipid, This blue color due to the reaction occurred between the anionic glycolipid biosurfactant with cationic CTAB methylene blue complex [14]. An alternative approach previously developed by [28,29] for the detection of extracellular rhamnolipids and other anionic glycolipids were employed for the screening of rhaminolipid biosurfactants production by *Pseudomonas sp.* and *Pseudomonas putid*a.

3.1.4 Detection by FTIR

The spectrum of FTIR analysis for biosurfactant extracted from *p.putida* showed in fig (1), the absorption bands at two regains 3406.05 cm^{-1} and 3346.27 cm^{-1} indicated the presence of free hydroxyl groups (-OH) due to H-bonding of polysaccharides and –OH stretching of carboxylic acid group, while the absorption band observed at 2929.67 cm^{-1} confirmed the presence of C-H stretching vibrations of hydrocarbon chain of alkyl groups (CH2-CH3). Also the Carbonyl stretching (C=O)bond at 1722.31 cm^{-1} was characteristic for ester compounds. The spectrum showed absorption bands at1452.30 cm^{-1} and 1234.36 cm^{-1} indicate the stretching bands carbon atoms with hydroxyl groups in the structure of sugar moiety, also the band at 1031.85 cm^{-1} was associated with the stretching vibrations of glycosidic linkage. The FTIR spectra of the biosurfactant was nearly to those reported for other glycolipid biosurfactant produced by *Pseudomonas otitidis* and *Pseudomonas aeruginosa* [30,31]. According to this results it can be concluded that the biosurfactant is glycolipid nature.

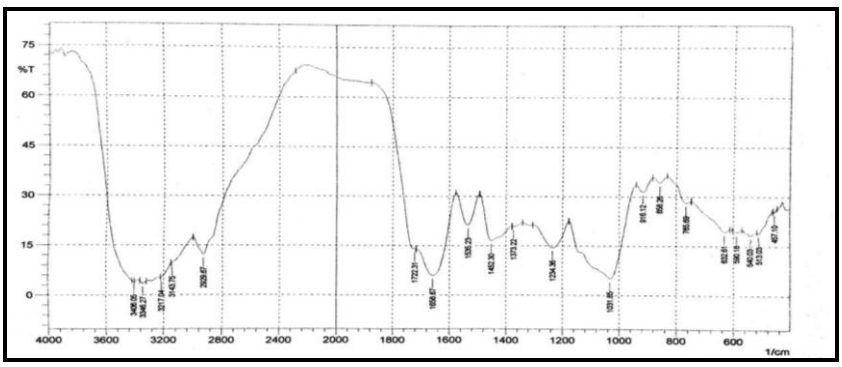

Figure (1) FTIR analysis of biosurfactant extracted from *pseudomonas putida*.

3.2 Stability of biosurfactant

The applicability of biosurfactant in several fields depends on their stability at extreme environmental conditions such as: temperature, pH and salinity. Therefore the effect of temperature on stability of biosurfactanat was studied by incubating the biosurfactant at various temperature between 20-100˚C for 30min. and measured the emulsification index E24%. it has been found that the emulsification index remained constant between 20-70˚C (fig.2).These results corroborate with those obtained by [32]who found that the emulsifying activity of the biosurfactants produced by *Pseudomonas fluorescens* remains stable after thermal treatment at 100ºC for 15 min, also [33] showed that the biosurfactant produced by *Pseudomonas putida* was thermostable, because the emulsifying activity was maintained even at the higher temperature of 100ºC, while the maximum activity 73% was observed at 40ºC, whereas the biosurfactant produced by *Pseudomonas aeruginosa* was found to be stable at temperature up to 120◦C [34].

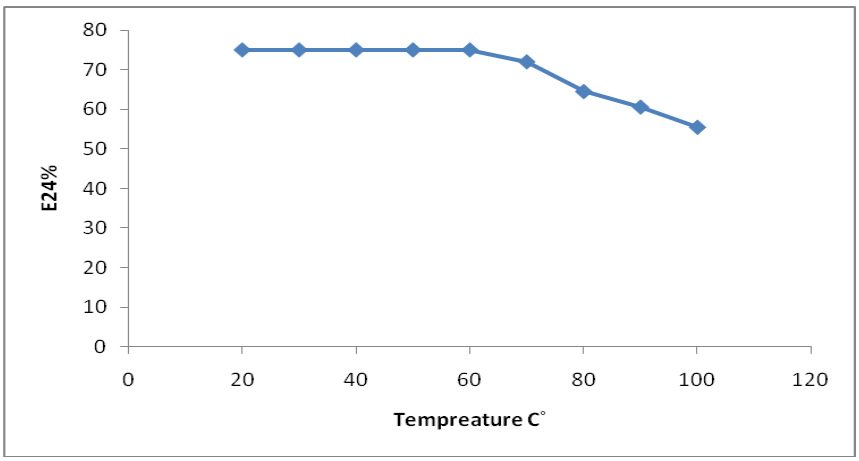

Figure (2):Effect of different temperatures on the biosurfactant stability

The effect of pH on stability of biosurfactant achieved in different solutions which adjusted to pHs values between 2-11, then the emulsification index was measured. Results showed that the highest emulsification index appeared in pH7 (74.6%), while in pH 5 and lower the emulsification index decreased, this suggesting that the biosurfactant was not stable below pH 5(acidic conditions). Also the results indicated that the E24% remained stable till pH 9 as in (fig. 3).When the pH increases from 5 to 8, the emulsification activity was increased, due to the negative charge of the polar head increases which lead to an increase solubility for biosuractant[35].At higher and lower pH values the emulsification activity of biosurfactants decreased, may be due to the occurrence of some alteration in the biosurfactant structure at these pH values. The rhamnolipids have their optimum aqueous solubility at neutral to alkaline pHs, which is refer to their acidic nature, the reported pKa is 5.6 [36]. The stability loss at low pH scale <5 is probably due to the occurrence of precipitation, caused by the consequent insolubility of the biosurfactant produced by *Pseudomonas aeruginosa* at these pH values [37,38]. In a previous studies achieved by [30,33] they proved that the maximum activity of biosurfactant produced from *Pseudomonas otitidis* and *Pseudomonas putida* at pH 7.

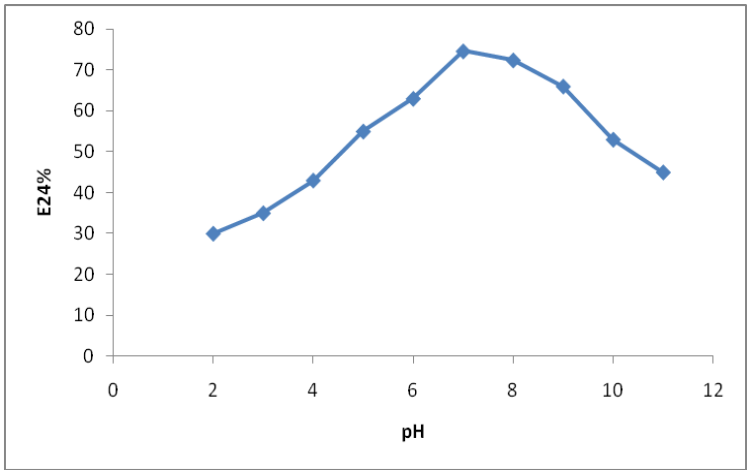

Figure (3): Effect of different pHs values on biosurfactant stability

The results of E24% showed that the biosurfactant gave good stability at concentrations 5, 10 and 15% of NaCl, while the E24% value decreased to 51.2% at concentration 20% as in (fig.4). In a previous study [39] they founded that biosurfactant of *P.putida* exhibited good resistance to NaCl concentrations up to 10g/l. As a result, a decrease in surface tension values is observed, probably because of the formation of a close-packed monolayer, caused by the fact that the negative charge is shielded by Na+ in the electrical double layer in the presence of NaCl [36, 40]. [41] they found that the biosurfactant of *Pseudomonas aeruginosa* was not significantly influenced by NaCl concentrations ranging from 0 to 20%, while [42] has been observed that the biosurfactant obtained from a marine *Pseudomonas aeruginosa* was tolerant to high salt concentrations (up to 35%), in other study little changes were observed in the activity of biosurfactant produced from *P.putida* between 1-7% salt concentrations[33].

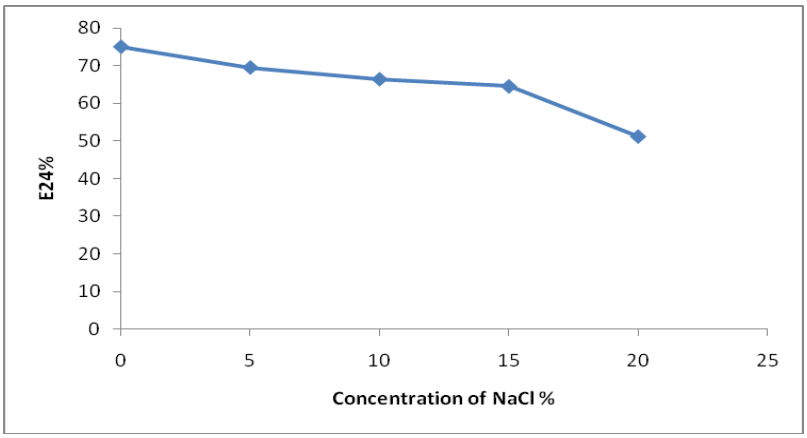

Figure (4): Effect of sodium chloride salt on the biosurfactant stability

IV. Conclusion

The results obtained from the present study indicated that the biosurfactant produced by *P. Putida* **PS6** isolated from contaminated soil was glycolipid in nature. The biosurfactant exhibited high emulsification activity and stability in a wide range of temperature, salinity and at neutral conditions, which makes it suitable for various industrial applications such as food, pharmaceutical and cosmetics industries.

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