Detection of Rhamnolipid Production in Pseudomonas aeruginosa

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Abstract. Large variety of microorganisms produce potent surface-active agents, biosurfactants, which vary in their chemical properties and molecular size. Rhamnolipids are naturally occurring glycolipids produced by Pseudomonas aeruginosa species of bacteria. The enormous diversity of biosurfactants makes them an interesting group of materials for application in many areas such as agriculture, public health, food, health care, waste utilization, and environmental pollution control such as in degradation of hydrocarbons present in soil. Their ability to reduce surface tension is a major characteristic of surfactants, which is the key ingredient used in detergents, shampoo, toothpastes. Four strains of Pseudomonas aeruginosa obtained from oil contaminated soil with diesel, kerosene or benzene using four detection methods: Blood hemolysis, Oil spreading technique, cetyl trimethylammonium bromide (CTAB) agar plate, and Emulsifying activity. Ten out of eleven strains tested demonstrated rhamnolipid production in one or more of the techniques used and with variable amounts, with Pseudomonas aeruginosa strain D and T displaying positive and high productivity in all methods used. A new modification of oil spread technique was performed in the present study as this method was found to be the most efficient, easiest, and reliable among the others.

Keywords: isolation, detection, rhamnolipids, Pseudomonas, oil contaminated soil,

Introduction

Biosurfactants are extracellular macromolecules produced by bacteria, yeast and fungi and in particular by natural and recombinant bacteria when grown on different carbon sources. Microbial surfactants have gained attention in recent years due to their commercial importance, diverse desirable characteristics such as biodegradability, selectively effectiveness, low toxicity, ecological acceptability and their ability to be produced from cheaper substrates (Raza et al., 2005) and (Rashedi et al., 2006). Furthermore, Rhamnolipids are found in the sputa of cystic fibrosis patients and can inactivate tracheal cilia of mammalian cells, and are involved in fluid-channel formation indicating that they are virulence factors. (Boles et al., 2005, Espinosa-Urgel, M. 2003). The use and potential commercial application of biosurfactants in the medical field has increased during the past decade. Their antibacterial, antifungal and antiviral activities make them relevant molecules for applications in combating many diseases and as therapeutic agents. In addition, their role as anti-adhesive agents against several pathogens indicates their utility as suitable anti-adhesive coating agents for medical insertional materials leading to a reduction in a large number of hospital infections (Rodrigues et al., 2006).
These molecules have tremendous potential for application in the bioremediation of soil and sand (Van Dyke et al., 1991), in the cleanup of hydrocarbon contaminated groundwater and enhanced oil recovery (Ron and Rosenberg, 2001), or wide variety of industrial processes involving emulsification, foaming, detergency, wetting, dispersing or solubilization (Desai et al., 1997). Biosurfactants, produced by a wide variety of microorganisms, have very different chemical structures and surface properties. It is therefore reasonable to assume that different groups of biosurfactants have different natural roles in the growth of the producing microorganisms. Moreover, as their chemical structures and surface properties are so different, each emulsifier probably provides advantages in a particular ecological niche. Several bioemulsifiers have antibacterial or antifungal activities. Bioemulsifiers also play an important role in regulating the attachment-detachment of microorganisms to and from surfaces. In addition, emulsifiers are involved in bacterial pathogenesis, quorum sensing and biofilm formation (Ron et al., 2001).

Pseudomonads produce extracellular lipids (biosurfactant) known as rhamnolipids. These amphipathic molecules shown in figure 1 are composed of hydrophobic lipid and one or two hydrophilic rhamnose units which provide these molecules with tensioactive properties capable of reducing surface tension, forming emulsions, and causing pseudosolubilization of insoluble substrates (Beal et al., 2000).

![Figure 1. Chemical structure of rhamnolipid.](image)

**Materials and Methods**

**Sample Collection and Processing:**

For the isolation of biosurfactant producing bacteria, oil contaminated soil samples (benzene, diesel or kerosene), were collected from a gasoline station where the oil spilled in the soil in Almajmooa, Mosul with a depth of 2-3 inches from the ground level using clean spatula (Anandaraj et al., 2010). Soil samples were transferred into sterile plastic containers and stored at 4°C until use. Soil samples (10 g) were suspended in 100 ml of sterilized nutrient broth, incubated in a shaker at 200 rpm for 48hrs to ensure complete mixing of soil and subsequently 1 ml of bacterial suspension was streaked on cetrimide agar plates (a selective medium for *Ps. aeruginosa*). The inoculated plates were incubated at 37°C for 24 hrs and observed for pigmented colonies. Seven clinical strains obtained from the Biology Department/ college of science/ University of Mosul were also cultivated on cetrimide agar. All isolated colonies were characterized as *Ps. aeruginosa* by colony morphology, oxidase test and growth on the selective cetrimide agar.
Production and Screening of Biosurfactants

The isolated culture (*Ps. aeruginosa*) was inoculated in 100 ml MS (Mineral Salt medium): glycerol 20 ml, KH$_2$PO$_4$ 0.7 gm, Na$_2$HPO$_4$ 0.9 gm, NaNO$_3$ 2 gm, MgSO$_4$.7H$_2$O 0.4 gm, CaCl$_2$.2H$_2$O 0.1 gm, Cetyltrimethylammonium bromide (CTAB) 0.2 gm, methylene blue 0.005 gm, 2 ml trace elements solution (acidified with 37% HCL): (NH$_4$)$_6$ Mo$_7$O$_24$.H$_2$O 0.6 gm, FeSO$_4$. 7H$_2$O 2 gm, MnSO$_4$. H$_2$O 1.5 gm. With final pH 6.7 and kept in shaker at 200 rpm/min for 1 week to ensure complete aeration at 35°C.

Biosurfactants from *Ps. aeruginosa* were screened using the following four methods:

1- Oil spreading method. Oil spreading technique was carried out according to the method described by (Youssef *et al*., 2004) and (Plaza *et al*. 2006). Briefly, fifty millilitre of distilled water was added to the petri dishes (15 cm diameter) followed by addition of 100 l of crude oil to the surface of water. Then 10 l of cell free culture broth was dropped on to the crude oil surface. The diameter of clear zone on the oil surface was compared to 10 l of distilled water as negative control. The spreading of oil drop was captured using digital camera.

A new modification was applied in this study in order to demonstrate a more defined and clear halo by staining the sunflower oil with sudan black or eosin. This gave a better view of the clear zone or halo.

2- Hemolytic Activity: Isolated strains were screened on blood agar plates containing 5% (v/v) human and rabbit blood and incubated at 37 °C for 24-48 h. Hemolytic activity was detected as the occurrence of a defined clear zone around a colony (Carrillo *et al*., 1996)

3- CTAB agar plate assay: This is a semi- quantitative assay for the detection of extracellular glycolipids or other anionic surfactants. It was developed by (Siegmund *et al*., 1991). The isolated bacteria are cultivated on a light blue mineral salt agar plate containing the cationic surfactant cetyltrimethylammonium and the basic dye methylene blue.

4- Emulsification Measurement: Emulsification activity was measured according to the method of Cooper *et al*., 1987 with a slight modification. To 2 ml of culture supernatant or biosurfactant crude extract, 2 ml of sunflower oil were added and vortexed at high speed for 2 min. The mixture was allowed to stand for 10 min prior to measurement. The emulsification activity is defined as the height of the emulsion layer divided by the total height and expressed as percentage (Thenmozhi *et al*., 2010).

Results and Discussion:

Screening for rhamnolipid producing *Pseudomonas aeruginosa* from oil-contaminated soil resulted in the recovery of 4 candidate isolates, also 7 clinical strains which were obtained from the Department of Biology /University of Mosul were used in the present study. All strains were oxidase positive, (figure 2)
Figure 2. Oxidase positive reaction of *Pseudomonas aeruginosa* strains.

Detection of rhamnolipid production by these isolates using the four methods mentioned previously revealed that 10 out of 11 strains gave positive results for at least two detection tests except one strain isolated from kerosene contaminated soil which responded weakly to the CTAB detection method.

Table 1 summarizes the response of all strains to the four different detection methods used as follows: 91% of the strains gave positive results in oil-displacement and emulsification methods, 75% responded positively to CTAB test and 63.6% had hemolytic activity in blood agar.

| Strain | Oil Displacement | Emulsification activity | Bld Hemolysis | CTAB |
|--------|------------------|-------------------------|---------------|------|
| K      | -                | -                       | -             | +(weak) |
| IPK    | +                | 83.3%                   | Nd            | -    |
| D      | ++++             | 67.5%                   | +             | +++  |
| B      | ++++             | 62.5%                   | -             | +( weak) |
| 3      | +                | 40%                     | ++            | ++   |
| CHL    | ++               | 92.5%                   | ++            | ++   |
| T      | +++              | 95%                     | ++            | +    |
| 9      | +                | 62.5%                   | ++            | ++   |
| Hosp   | +                | Nd                      | ++            | ++   |
| 59     | ++++             | 83.3%                   | -             | -    |
| 2      | +                | 80%                     | ++            | +    |

1- Oil-displacement method: Results showed that Bacteria strains D, B (soil samples), T, and 59 (clinical samples) exhibited the highest oil clearing zone and oil displacement area. This method shown in figure 3 depends on the decrease in water-oil interfacial tension caused by the biosurfactant (Morikawa et al., 2000).
Control (+) (++) (+++)

**Figure 3.** Zone formation by rhamnolipid producing strains in Oil- spreading method.

**Figure 4.** Modified rhamnolipid detection method (sudan black stained sunflower oil).

**Figure 5.** Modified rhamnolipid detection method (eosin stained cell -free culture).

The oil spreading method is rapid and easy to carry out, requires no specialized equipment and just a small volume of sample. It can be applied when the activity and quantity of biosurfactant is low (Plaza *et al.*, 2006) and (Youssef *et al.*, 2004) demonstrated that the oil spreading technique is a reliable method to detect biosurfactant production by diverse microorganisms. Ten out of eleven (91%) of the strains gave positive results with variation in halo diameter. This is explained by the direct relationship between the diameter of the sample and concentration of the biosurfactant and in contrast, the drop lacking biosurfactant remains beaded due to the hydrophobicity of the oil surface that cause aggregation of droplets (Nast *et al.*, 2009).

Our new modification was made on this method in order to clarify the zone by staining the sunflower oil used with sudan black stain. Staining gave a more profound and clear black boarders of the oil drop and consequently gave a better view as the oil drop spread on the surface, figure 4. Eosin, on the other hand was used to stain cell free culture, figure 5, which also gave a better view, however, the sudan black stain was more efficient to give a better view.

2- Hemolytic Activity: Rhamnolipids in the right concentration are a hemolysin, meaning that they can break up red blood cells by lysis, destroying the blood cell wall (Johnson *et al.*, 1980) (Fujita *et al.*, 1980).
1988) (Carrillo et al., 1988). The hemolytic activity was determined by the occurrence of a defined clear zone around the colony as shown in figure 6.

![Figure 6. B-hemolysis activity by rhamnolipid producing Pseudomonas aeruginosa strains in blood agar.](image)

As 20% of the best rhamnolipid producer strains did not completely lyse blood, the ability of producer strains for hemolytic may not be trustworthy. This is in accordance with many workers who demonstrated that it is not a reliable method to detect biosurfactant production due to many bio-products that can cause red blood cell lysis which do not necessarily have to be surface active molecules (Youssif et al., 2004) and (Grayna et al., 2006).

3- CTAB Method: If anionic surfactants are secreted by the microbes growing on the plate, they form a dark blue- purple, insoluble ion pair with cetyltrimethylammonium bromide and methylene blue. Accordingly, rhamnolipid-producing strains can be recognized by the dark blue halos formed around the colonies, figure 7, and, ideally, the amounts of rhamnolipids produced could be correlated with the areas of the halos (Siegmund, et al., 1991).

This method is specific for glycolipids and has been used for strain selection and screening studies, particularly for differentiating rhamnolipid producers from non-producers. This method is specific for glycolipids and has been used for strain selection and screening studies (Gunther et al., 2005) and (Wild et al., 1997), particularly for differentiating rhamnolipid producers from non-producers.

However, the method is often not quantitative enough for selecting the top producer among multiple producing strains, especially when many of them also produce other pigments that interfere with the blue. Therefore, plates were incubated after inoculation for 48 h at 34°C and then stored at 4 °C for at least 24 h (typically 48 h). The cold storage tended to darken the blue color, making visible recognition less difficult (Gunther et al., 2005) and (Neissa 2009).
Figure 7. CTAB assay showing the dark blue-purple halo around the rhamnolipid producing strains.

4- Emulsification Measurement: One of the criteria for selection of potent isolates was emulsification capacity of more than 2-fifty percent. (Afshar et al., 2008).

The emulsification activity is defined as the height of the emulsion layer divided by the total height and expressed as percentage.

According to our data, 9 rhamnolipid producing strains showed emulsification activity varying from 62-95%. Strain T was a very potent emulsifier in addition to strain 59 which was exceptional in foaming activity, figure 8.

Figure 8. Emulsifying activity of *Pseudomonas aeruginosa* strains using sunflower oil.

Screening for rhamnolipid producing *Pseudomonas aeruginosa* from oil-contaminated soil resulted in the recovery of 4 candidate isolates as these samples are known habitat and source of versatile
microorganisms and since the microorganisms capable of emulsifying and solubilizing hydrophobic agents have an apparent advantage over their competitors, sampling of this nature provides a source rich in microorganisms with desired characteristics. (Okerentugba et al., 2003) stated that microbiological communities exposed to hydrocarbons will adapt to this exposure through selective enrichment and genetic changes resulting in an increase in hydrocarbon-degradation. According to Ron et al., 2001, biosurfactants can fulfill various physiological roles and provide different advantages to their producing strains:

- increase the surface area of water-insoluble substrates by emulsification,
- increase the bioavailability of hydrophobic substrates,
- bind heavy metals,
- be involved in pathogenesis,
- possess antimicrobial activity,
- regulate the attachment/detachment of microorganisms to and from surfaces.

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