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The Ability of Some Pseudomonas Strains to Produce Biosurfactant

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Abstract

This study aims to identify the capacity of two fluorescent *Pseudomonas* plant growth-promoting rhizobacteria and one strain of *P. aeruginosa* to produce biosurfactants. The strains were grown in Mineral Salt Medium (MSM) with 1% (v/v) tapis gas oil as a sole carbon and energy source. Biosurfactant synthesis was monitored by measuring surface tension, emulsifying index (E24), drop-collapse and bacterial adhesion to hydrocarbons. The best strain, *P. aeruginosa*, was able to reduce the surface tension to 55 mNm-1 with an E24 of 25.29%. The hydrophobicity was below 30%, a significant decrease for *P. aeruginosa* was signaled. Bacterial adhesion to hydrocarbons assay results showed that, *P. aeruginosa* had the highest level of cell adhesion (25.4%), followed by *P. fluorescens* (22.90%) and *P. putida* (17.07%). Temperature of 30°C and pH 7 were found to be optimum. This study showed that these PGPR strains had the ability to biodegrade gas oil and concurrently produce biosurfactant.

Keywords: Biosurfactants; Tapis gas oil; *Pseudomonas*; Plant growth-promoting bacteria

Introduction

Environmental oil pollution is a common phenomenon that causes significant ecological and social problems. Moreover, for a variety of reasons, the traditional treatment processes used to decontaminate polluted areas have been limited in their application [1]. The biodegradation of petroleum-contaminated soil has become a very important issue in environmental protection over the last two decades [2], mainly because of the possibility of using microbial strains, which significantly reduces the costs of soil cleanup technologies. This microbial bioremediation has received increasing attention because of a number of advantages over other remediation technologies. However, only 1% of the overall microbial population can degrade crude oil and its derivatives. *Pseudomonas* spp. belongs to a genus with very high gas oil biodegradation activity. The isolation, identification and characterization of crude oil degrading bacteria and their remediation efficacy from different ecological niches has previously been reported by several other researchers [3,4]. Thus, there is a growing interest in the use of microorganisms for reduction of organic pollutants [5]. To date, the most important organic degrading bacteria have been assigned to the *Pseudomonas* genera. Some of them have been widely used for bioremediation of oil-contaminated environments [6]. This capacity of bioremediation is a consequence of several mechanisms including the production of a wide range of metabolites such as siderophores and biosurfactants. In addition, *Pseudomonas* spp. are able to degrade alkynes and/or aromatic hydrocarbons under aerobic or anaerobic conditions [7].

The purpose of the present study is to assess the ability of certain fluorescent pseudomonads (strains of *P. fluorescens, P. putida, P. aeruginosa* and a consortium of these strains to degrade gas oil by producing biosurfactants and siderophores.

Methods

For the gas oil biodegradation study, bacterial strains that were employed include a PGPR (plant growth-promoting rhizobacterium) (*P. fluorescens* (P9) and PGPR P. putida (P10),and the strain *P. aeruginosa* (PR) which was isolated from contaminated surface soil. P9 and P10 strains were isolated from *Triticum* spp. *rhizosphere* of the region of Mascara (Northern-Algerian West, 2°,11'W, 35°, 26 'N). The results of the biochemical characterization, determined by means of API 20NE; bio Merieux Vitek strips and on the basis of *Pseudomonas* biochemical tests as described in Bergy's Manual of Determinative Bacteriology , permitted the identification of these strains as *P. fluorescens, P. putida,* and *P. aeruginosa.* The fluorescent strains were Gram-negative, aerobic, , catalase and oxidase positive rods, showed oxidative metabolism on Hugh Leifson medium and grew at 4°C; only PR grew at 41°C. *P. fluorescens* and *P. putida* had the ability to produce plant growth regulator (IAA) (89 $\mu g l^{-1}$ and 116 μg^{1-} respectively), siderophores and solubilize phosphate.

Use of gas oil as a carbon and energy source

The *Pseudomons* strains from overnight cultures (10^4-10^5 cells/ml) were transferred to 100 ml of sterile mineral medium (MM) described by Abu-Ruwaida et al. [8] with 0.2% (v/v) of gas oil as carbon and energy source. The cultures were grown aerobically at 30°C for 7 days with shaking (150 rpm). Bacterial growth was estimated by two parameters: colony forming unit (CFU/ml) (for strains) and optical density of the cultures at 600 nm (for bacterial consortia).

Gas oil biodegradation

The bacterial strains from overnight cultures (10^4-10^5 cells/ml) were transferred to 250 ml Erlenmeyer flasks, each containing different concentration of gas oil (20, 40,60, 80,100 and 120%) and 100 ml of sterile Mineral Medium MM (containing in gl^{-1}):L-asparagine,05; K_2HPO_4,05; $MgSO_4 \cdot 7H_2O$,0.2; $FeCL_3 \cdot 6H_2O$,0.02). The pH of the medium was adjusted to 7.0 ± 0.2. Cultivations were performed in 250

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ml flasks containing 50 ml medium at room temperature 28 ± 2, and stirred in a rotary shaker at 150 rpm for five days.

Influence of pH and temperature on the growth and degradation of gas oil: Mineral medium (MM) with 0.2% (v/v) of gas oil was prepared at pH 5 to 10 using 1 N HCl and NaOH. To maintain the pH, citrate–phosphate buffer (pH 4-6), phosphate buffer (pH 7 and 8), and carbonate–bicarbonate buffer (pH 9 and 10) were used as described by [9]. The cultures were grown aerobically at 30°C for 7 days with shaking (150 rpm). The influence of temperature (20-40°C) was studied using the same experimental condition of culture at pH 7.

Biosurfactant production kinetics

Till date, there are nine different screening methods described by the scientific literature about biosurfactant production such as hemolytic assay, bacterial adhesion to hydrocarbons (BATH) assay, drop collapse assay, oil spreading assay, emulsification assay, surface tension measurement, titled glass slide test, blue agar plate and hydrocarbon overlay agar assay [10].

In the present investigation the kinetics of biosurfactant production were followed in batch cultures for 96 hours at optimum conditions by measuring the surface tension, emulsification index E24 of supernatant samples obtained after cell separation and testing drop collapse assay.

Surface tension measurement: The surface tension measurement(s) of cell free supernatant was determined in a tensiometer (Krüss), using the method of Abu-Ruwaida et al. [8]. The values reported are the mean of three measurements. All measurements were made on cell-free broth obtained by centrifuging the cultures at 10,000 x g for 25 min.

Emulsification index (E24): The E24 of culture samples was determined by adding 2 ml of gasoil to the same volume of bacterial culture, mixing with a vortex mixer for 2 min, and allowing the mixture to stand for 24 hours. The E24 index is given as percentage of height of emulsified (he) layer (mm) divided by total height of the liquid column (ht) (mm) [11] according to the formula below:

$$E24 = (he / ht) \times 100$$

Qualitative evaluation of biosurfactant production: The drop-collapse method was used for initial identification of biosurfactant-producing bacteria [12]. Briefly, 5 µl of 1.8 mineral oil (Vaseline) was added to each well of a 96 well microtiter plate lid. The lid was equilibrated for 24 h at room temperature, and then 10 µl of the culture was added to the surface of the oil. The shape of the drop was inspected after 1 min; if the drop remained beaded, the result was scored as negative. If the drop collapsed, the result was scored as positive. Tests were carried out intriplicate using culture supernatant and cell suspensions. Sodium dodecyl sulfate (SDS) and Hexadecyltrimethylammonium (HDTMA) were used as positive controls while distilled water and MM as negative controls.

Bacterial adhesion to hydrocarbons (BATH) assay: Microbial surface hydrophobicity was assessed by the Bacterial Adhesion to the Hydrocarbon Method (BATH) described by Rosenberg et al. [13] with modifications. The culture was grown on different carbon sources including gas oil and a mixture of dodecane, hexadecane and gas oil. Cells in exponential phase were centrifuged at 7000×g for 4 min, washed twice with phosphate urea magnesium buffer (in gL^{-1}: K_2HPO_4,19.7; KH_2PO_4, 7.26; H_2NCONH_2, 1.8; and $MgSO_4 \cdot 7H_2O$, 0.2), and suspended to an OD_{600} of approximately 1.0 (A_0–initial OD_{600}). Next, 500 µl of gas oil was added to 5 ml of microbial suspension and vortexed for 2 min at 2500 rpm. After 10 min, the OD_{600} of the aqueous phase was measured (A_1). The degree of hydrophobicity is calculated as $[1-(A_0-A_1)/A_0]\times100\%$. Each experiment was tested in triplicate. When hydrophobicity is between 0% and 30%, it is assumed that the cell surf ace of the microorganism has hydrophilic properties; from30% to 40%, the surface has mixed hydrophobic and hydrophilic properties; above 40%, the cell surface of the microorganism has hydrophobic properties [14].

Statistics

All experiments were conducted in triplicate. Results were evaluated for statistical significance using *Pearson* correlation to determine how these variables were interrelated.

Results and Discussion

Use of gas oil

All three of the bacterial strains utilized gas oil as a sole source of carbon and energy (Figure 1). The greatest specific growth rate was observed for *P. aeruginosa* (PR), this growth rate increases with an increase in the gas oil concentration. However, for *P. fluorescens* (P9) the growth rate apparently declined at 80% gas oil. It is interesting to note that growth of *P. aeruginosa* remained significant at high concentrations of gas oil. This indicates that the isolate has a novel ability to resist inhibition by this substrate. This is shown by the high

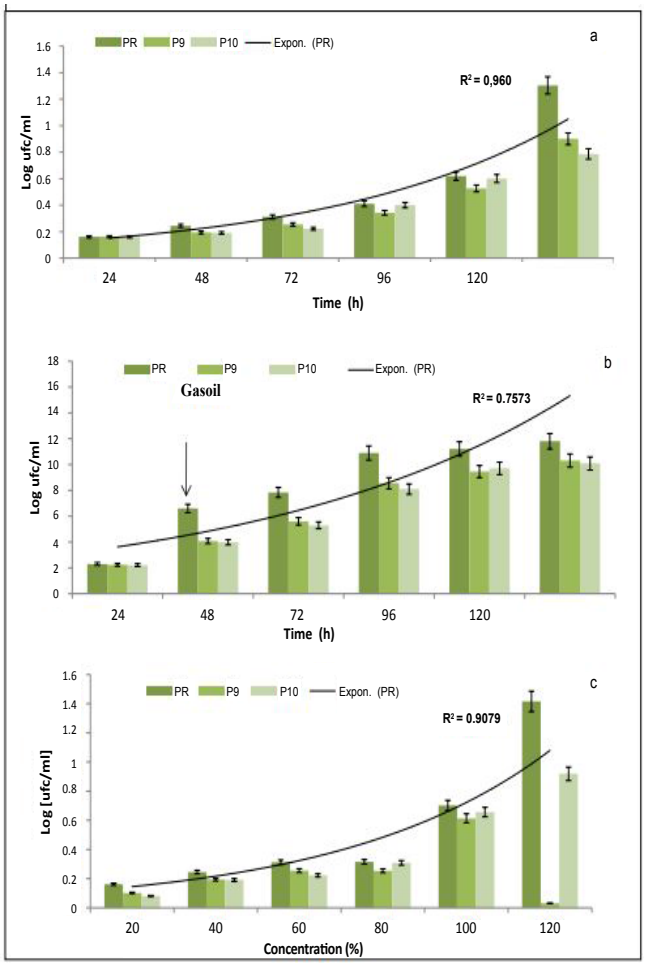

Figure 1: The bacterial growth curves in the presence of Gas oil (a,b) and with different concentrations (c).

coefficients of correlation for PR strain (R^2=0.757; R^2=0.907). The R^2 gives an indication of the degree of bio-remediation of gas oil.

The most prevalent bacterial hydrocarbon degraders and surfactant producers, belong to the genera are *Pseudomonas, Achromobacter, Flavobacterium, Micrococcus, Bacillus, Arthrobacter, Klebsiella, Acinetobacter, Aeromonas, Alkaligenes, Streptococcus sp, Corynebacterium sp, Moraxella sp,* and *proteobacteria* [15]. Rhamnolipids from *Pseudomonas* are the best known glycolipid surfactants, and their potential applications range from uses in cosmetics, food, pharmaceuticals, paper, metal and ceramics, to environmental uses such as in bioremediation [16].

It is known that *P. aeruginosa* strains are able to produce six types of rhamnolipids, which possess similar chemical structure and surface activity [17]. *P. aeruginosa* strains are commonly isolated from sites contaminated with hydrocarbons around the world, and have already been used in bioremediation procedures [18].

The study of hydrophobicity and gas oil biodegradation with three bacterial consortia was also carried out (Figure 2). The results obtained indicated that these consortia could efficiently degrade gas oil. Sugiura et al. [19] reported that biodegradation caused by mixed cultures was more effective than that caused by pure cultures mainly due to the complexity of oil products. Deppe et al. [20] reported that an arctic microbial consortium was able to degrade 77% of crude oil from source-I and 71% of crude oil from source-II at 20°C. This validates the use of a microbial consortium for biodegradation of complex mixtures of hydrocarbons in crude oil [9]. These observations corroborate those of Alkhatib et al. [21] who concluded that a bacterial consortium showed a good degradation rate of Total Petroleum Hydrocarbon (TPH) which suggests the potential application of the consortium for soil bioremediation.

Studies on the effect of the pH and temperature showed that pH 7 was favorable for the bacterial isolates and mixed bacterial (Figure 3a). Hence the pH 7 was selected; the bacterial isolates showed maximum gas oil degradation at 30°C (Figure 3b). Sathishkumar et al. [9] have reported pH 7 as the optimal range for hydrocarbon degradation. Extremes in pH were shown to have a negative influence on the ability of microbial populations to degrade hydrocarbons [22]. At low temperatures, the viscosity of the oil is increased, volatilization of alkanes reduced, and the water solubility decreased, delaying and decreasing the onset of biodegradation [23]. Banat et al. [22] reported 30°C to be the optimum temperature for microbial growth and PAH

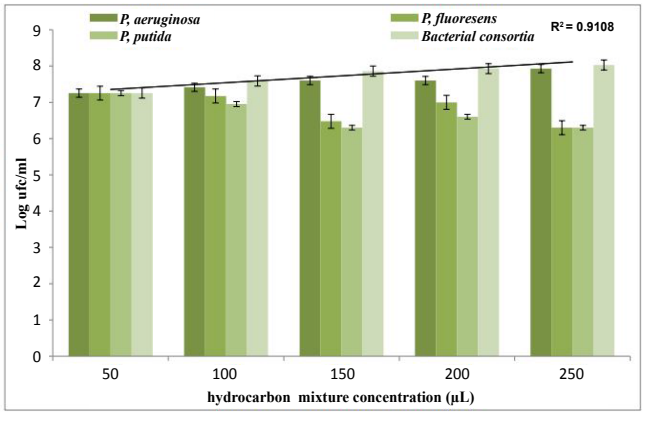

Figure 2: The bacterial growth curves in the presence of hydrocarbon mixture concentrations.

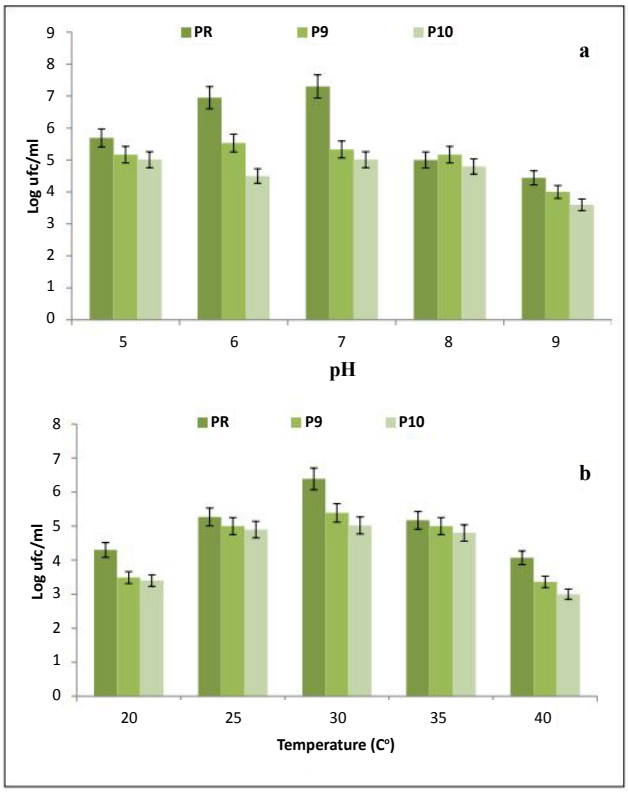

Figure 3: Effect of pH (a) and temperature on gas oil degradation (b).

degradation. The data corroborate those of [24], who found that using the mixed bacterial consortium, which can efficiently degrade the crude oil components, maximum degradation was achieved at a temperature of 30°C and pH of 7.5. Hence we suggest the use of the above optimised conditions and the mixed bacterial consortium for bioremediation of crude oil-contaminated sites.

Surface tension

Biosurfactants are surface active compounds produced by microorganisms. These molecules reduce surface tension between aqueous solutions and hydrocarbon mixtures [23] and enhance the bioavailability of hydrophobic substrates to bacterial cells. Reduction in the surfactant tension of the medium was a result of emulsification of gas oil by the surfactant produced by these fluorescents strains. Due to their amphiphilic nature in possessing both polar and non-polar domains, biosurfactants are able to partition preferentially at the interface between phases of different degrees of polarity and hydrogen bonding such as water oil, water air or solid water interfaces and are thus able to reduce the interfacial or surface tension [13,22].

There was, therefore, a significant lowering ($p<0.01$) of the surface tension of the culture supernatants. The decrease in surface tension indicated the production of extracellular surface active compounds using the same carbon source. Indeed, the microbial compounds that exhibit particularly high surface activity and emulsifying activity are classified as biosurfactants. These are structurally diverse surface active compounds capable of reducing surface and interfacial tension at the interfaces between liquids, solids, and gases, thereby allowing them to mix or disperse readily as emulsions in water or other liquids [25]. As shown in Figure 4, all the isolates were able to lower the surface tension,

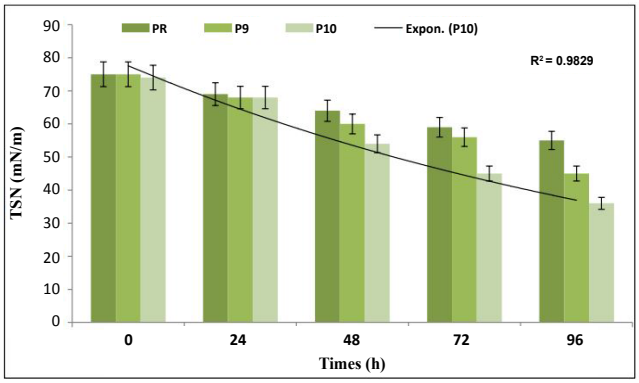

Figure 4: Surface Tension evolution of Culture supernatant.

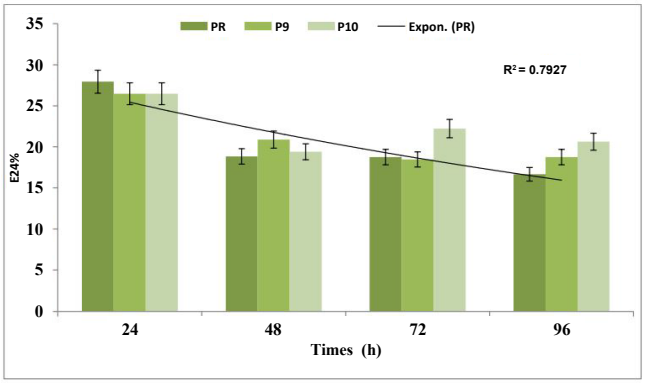

Figure 5: Emulsification Index evolution of Culture Supernatant.

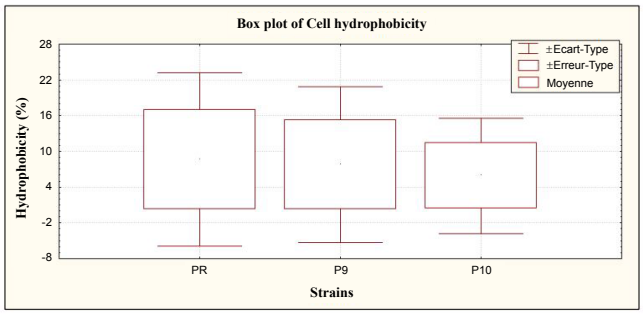

Figure 6: Cell hydrophobicity to Gas oil.

presumably via biosurfactant production. We observed the reduction of surface tension values from 75 mN/mto 55 mN/m, 45 mN/m and 36 mN/m, respectively for *P. aeruginosa, P. fluorescens* and *P. putida*. Surfactant production is a desirable characteristic in oil bioremediation, several *Pseudomonas* are able to synthesize biosurfactants of diverse chemical nature. The most studied of these compounds are the rhamnolipids produced by *Pseudomonas aeruginosa* [26]. Nitschke et al. [16] reported that glycolipids produced by *Pseudomonas* are low molecular weight compounds, which can lower the medium surface tension below 30 mN/m. These results corroborated the presence of tensoactive compounds secreted by the isolated strains. The tensoactive compounds have a high relevance in several industries including petrochemicals in which surfactants are used to enhance oil recovery [27]. It can be assumed that this will help to improve the accessibility and bioavailability of water-immiscible petroleum hydrocarbons [28].

Emulsification index (E24)

E24 was the method used to quantify the emulsification caused by the biosurfactants produced by the tested bacteria. As shown in Figure 5, all strains presented varying degrees of emulsification, indicating the production of biosurfactant compounds. The *P. aeruginosa* strain showed the best result, reaching an emulsification index of 27.94%. This value is in agreement with previous results. For example, Pruthi et al. [29] values found that cultures of *P. aeruginosa* growing on a similar medium presented an emulsification index value of 30%, and was considered an excellent producer of biosurfactant.

The drop-collapse and hemolytic assay

The three isolates were positive for the drop collapse activity and hemolytic assay. Carrillo et al. [30] found an association between hemolytic activity and surfactant production and they recommended the use of blood agar lysis as a primary method to screen biosurfactant production. None of the studies reported in the literature Satpute et al. [31] mention the possibility of biosurfactant production without hemolytic activity. However, in some studies the hemolytic assay excluded many good biosurfactant producers, and in some reports strains with positive hemolytic activity were found negative for biosurfactant production [10].

Cell hydrophobicity (BATH)

Theses isolates demonstrated a broad range of hydrophobicities. The hydrophobicity for the gas oil was below 30%; BATH assay results showed that *P. aeruginosa* had maximum cell adhesion with gas oil (25.4%), followed by *P. fluorescens* (22.90%) and *P. putida* (17.07) (Figure 6). Our results disagree with [14] data. The results of hydrophobicity analysis indicated that the modification of microbial cell surface depends on the metabolic of surfactant and microorganism genus. According to Zhang et al. [32], mutual attraction between biosurfactant and microbial cells can lead to an increase in cell hydrophobicity, and therefore, cells have better contact with the hydrophobic substrate and finally more biodegradation maybe achieved.

Interestingly, cell hydrophobicity is also an indication of biosurfactant production. Cell surface properties are important factors that determine the rate of degradation of hydrophobic substrates [33]. In an early investigation, cells exhibiting highest hydrophobicities were among the fastest hydrocarbon degraders [32].

Conclusion

Bioremediation using *Pseudomonas* strains with plant-growth-promoting traits offers an attractive treatment option because the technology is cost-effective and environmentally compatible. The work reported here demonstrates the biosurfactant-producing potential of an indigenous *P. aeruginosa,* and two PGPR *Pseudomonas* strains using gas oil as a carbon source. The findings in this study showed that PGPR *Pseudomonas* strains could be useful in hydrocarbon degradation and bioremediation. These strains presented desirable oil bioremediation characteristics like surfactant production, high tolerance to different hydrocarbons with great catabolic versatility. All of these characteristics make these strains interesting candidates as oil bioremediation agents.

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