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# Role of Rhamnolipids in Biofilm Formation and Velocity of *Pseudomonas aeruginosa*

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ABSTRACT

**Background:** Biofilm is described as an accumulation of microbial organisms connected to a living or unmoving surface mainly through self-secreted polymeric materials. With a complete understanding of biofilm behaviors and the role of rhamnolipids in its stability or dispersion, a new path could be designed in the treatment of infections like *Pseudomonas aeruginosa* (*P. aeruginos*a). The purpose of this study was to investigate the role and function of rhamnolipids in *P. aeruginosa* velocity and biofilm formation ability. .
**Materials & Methods:** In this study, 68 *P. aeruginosa* clinical samples were isolated from February 2022 to 2023 and confirmed based on culture and molecular methods. The presence of genes associated with di-rhamnolipid (*rhlC*) and mono-rhamnolipid (*rhlA* and *rhlB*) biosynthesis was detected by PCR method. For velocity assay, bacterial cultures on Bushnell Haas medium were monitored for 24 and 72 hours (0.5%).
**Findings:** The results showed that the distribution of biofilm strength among *P. aeruginosa* strains was normal. The frequency of *rhlC* was significantly different from those of *rhlA* and *rhlB* ($p$= .01). In the first 24 hours, the velocity of *P. aeruginosa* on Bushnell Haas with glucose was 2 µm/min and decreased during 72 hours. But after 72 hours, the velocity of moderate and weak biofilm-producing strains on solid medium with glycerol was constant.
**Conclusion:** In this study, rhamnolipids produced from different carbon sources showed different behaviors on colony shape, velocity, and strength of bacterial biofilms.

**Keywords:** *Pseudomonas aeruginosa*, Biofilm, Rhamnolipid, Velocity

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[1] Kaya E, et al. In vitro interaction of... [2] Zhou H, et al. Pyocyanin biosynthesis protects ... [3] Thi MT, Wibowo D, Rehm BH. Pseudomonas aeruginosa... [4] Tuon FF, Dantas LR, Suss PH, Tasca Ribeiro VS. Pathogenesis of... [5] Nickzad A, Deziel E. The involvement of rhamnolipids... [6] Shatila F, Diallo MM, Sahar U, Ozdemir G, Yalcin HT. The effect of carbon... [7] Chong H, Li Q. Microbial production of... [8] Schmidberger A, Henkel M, Hausmann R, Schwartz T. Expression of genes... [9] Reis RS, Pereira AG, Neves BC, Freire DM. Gene regulation of... [10] Othman Ahmad R, Ahmadi A, Bahmani N, Taherpour A. Application of... [11] Bernier SP, Ha DG, Khan W, Merritt JH, O'Toole GA. Modulation of Pseudomonas... [12] Köhler T, Curty LK, Barja F, Van Delden C, Pechère JC. Swarming of Pseudomonas... [13] Caiazza NC, Shanks RM, O'Toole GA. Rhamnolipids modulate... [14] Mishra I, Fatima T, Egamberdieva D, Arora NK. Novel bioformulations... [15] Karballaei Mirzahosseini H, et al.Meta-analysis of biofilm... [16] Yousefpour Z, Davarzani F, Owlia P. Evaluating of the... [17] Yakout MA, Abdelwahab IA. Diabetic foot ulcer ... [18] Shatti HH, Al-Saeed WM, Nader MI. Effect biofilm formation... [19] Davarzani F. et al. Evaluation of... [20] Abdelraheem WM, et al. Detection of biofilm... [21] Deziel E, Lepine F, Milot S, Villemur R. Mass spectrometry... [22] Soberon-Chavez G, Lepine F, Deziel E. Production of ... [23] Schuster M, Greenberg EP. Early activation of ... [24] Al-Tahhan RA, Sandrin TR, Bodour AA, Maier RM. Rhamnolipid-induced ... [25] Pamp SJ, Tolker-Nielsen T. Multiple roles of... [26] Davey ME, Caiazza NC, O'Toole GA. Rhamnolipid surfactant... [27] Abdel-Mawgoud AM, Lepine F, Deziel E. Rhamnolipids... [28] Ron EZ, Rosenberg E. Natural roles of... [29] Espinosa-Urgel M. Resident parking:... [30] Christova N, et al. Chemical characterization... [31] Moussa T, Mohamed M, Samak N. Production and...

## Introduction

*Pseudomonas aeruginosa* (*P. aeruginosa*) is known as an important pathogenic bacterium that could cause a wide range of diseases from wound infection to sepsis and life-threatening pneumonia in cystic fibrosis patients. This bacterium is involved in infections associated with implanted endovascular and urinary catheters and other medical devices used in modern medicine [1, 2]. Due to the widespread use of antibiotics, currently nearly 30% of *P. aeruginosa* clinical isolates are multidrug resistant and show resistance against many antibiotics. In particular, carbapenem-resistant *Pseudomonas* is one of the six most dangerous superbugs for human health. Since 2017, this bacterium has been considered by the World Health Organization (WHO) as one of the most common antibiotic-resistant pathogens that could develop resistance to new antibiotics [2].

One of the reasons for resistance to antimicrobials, inhibition of phagocytosis, and self-protection against environmental stresses, is the biofilm formation ability of *P. aeruginosa*, which increases its colony-forming capacity [3]. This ability is enhanced through effective communication with the quorum sensing (QS) phenomenon, which promotes effective cell-to-cell communication. Biofilm is observed in most patients with chronic lung infections, ulcers, rhinosinusitis, and other infections, so that up to 90% of the healing process of chronic ulcerative infections is weakened due to the formation of biofilm, which increases the health care and economic burden [4].

In recent years, many studies have been conducted on rhamnolipids as biofilm-stabilizing compounds. These compounds are involved in various stages of biofilm development, from the first stage of cell communication to the stability and dispersion/separation of the biofilm structure [5]. The biosynthesis of rhamnolipids by *P. aeruginosa* is done in exponential and stationary phases with nitrogen or iron deficiency [6]. Studies have shown that many bacterial agents carry almost the majority of enzymes involved in rhamnolipid production, except for rhlA, rhlB, and rhlC as the three key enzymes [7]. These key enzymes participating in rhamnolipid production are encoded by *rhlABC* gene of *P. aeruginosa*. Given the development of antibiotic resistance and emerging resistance that is faster than the design and introduction of new antibiotics into the field of treatment, a thorough understanding of bacterial behaviors, production of secretory compounds, and bacterial cell interactions seems to be useful in controlling incurable infections caused by bacteria such as *P. aeruginosa*. Although rhamnolipid is considered as a virulence factor of *P. aeruginosa* with surfactant properties, it has been reported that at high concentrations, it destroys the intercellular connections of sheep tracheal cells and stops the movement of cilia of airway epithelial cells; in addition, traces of rhamnolipid have been observed in serum and cerebrospinal fluid of Alzheimer's patients [9]. But currently, rhamnolipids are used in various industries,

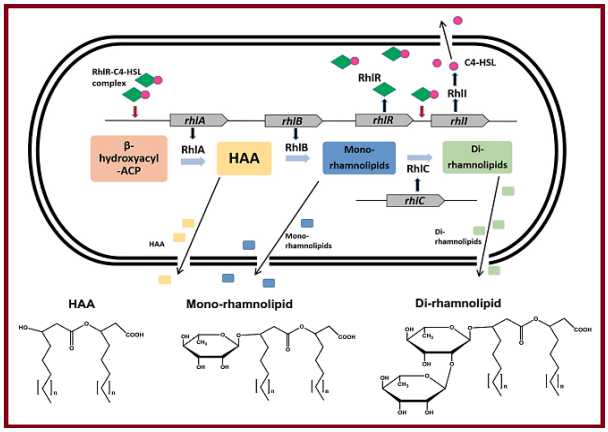


**Figure 1)** Rhamnolipid biosynthesis pathway by *P. aeruginosa*. *rhlAB* genes produce rhamnolipids after the interaction of the RhlR–C4-HSL complex with the *rhlA* promoter [8].

including pharmaceutical, medical, cosmetic, and agricultural industries; remediation of environmental contamination; etc. [6]. Therefore, with a complete understanding of biofilm behaviors, the role of rhamnolipids in biofilm stability or dispersion, and the expression of biofilm-related genes or controllers, a new pathway could be designed, especially in the treatment of *P. aeruginosa* infections.

**Objectives:** The purpose of the current study was to investigate the role and function of rhamnolipids in *P. aeruginosa* velocity and biofilm formation ability.

## Materials and Methods

**Specimen collection:** In this study, 68 *P. aeruginosa* clinical samples were collected from February 2022 to 2023 and confirmed by biochemical tests. The final confirmation test was polymerase chain reaction (PCR), which was performed by targeting the 16sRNA gene (135 bp) and using the following forward and reverse primers: -CTTACGGCCAGGGCTACACA- and -GCGATTCCGACTTCACGCAG-, respectively. The PCR reaction was performed with a total volume of 25 µL under the following thermal cycling program: a preliminary cycle involving a denaturation step at 94 ˚C for 3 min; followed by 35 cycles comprising denaturation at 94 ˚C for 60 s, annealing at 62 ˚C for 30 s, and extension at 72 ˚C for 60 s; and a final cycle involving an extension step at 72 ˚C for 10 min. PCR products were evaluated using agarose gel (1%) electrophoresis run at 100 volts for 45 min. In the current research, the standard strain *P. aeruginosa ATCC27853* with a strong biofilm formation ability was used as a positive control [10].

**Biofilm assay:** *P. aeruginosa* strains ($7.5\times10^5$ CFU) were sub-cultured in brain heart infusion (BHI) broth (1 mL) containing glucose (0.2%) at 37 ˚C for 24 hrs in microtubes. One tube with BHI without bacteria was used as a negative control (blank). After incubation, the suspensions were discarded, and the microtubes were subjected to three consecutive washes with water. For fixation, 1200 µL of methanol was added to each microtube (5 min). After discarding the methanol and drying at room temperature, 1200 µL of crystal violet (0.1%) was added to each tube for 15 min. Then the tubes were subjected to three consecutive washes with water and drying at room temperature, then 1200 µL of acetic acid (30%) was added to the tubes to solve the binding stain. Finally, 200 µL of the resulting solution was transferred to microplates, and the plates were read by an ELISA (enzyme-linked immunosorbent assay) reader at 595 nm wavelength. Based on the definition, biofilms fall into four categories: strongly adherent (4 ODc < OD), moderately adherent (2 ODc < OD < 4 ODc), weakly adherent (ODc < OD < 2 ODc), and nonadherent (ODc > OD) [11]. This method has been modified.

**Rhamnolipid genes:** Bacterial DNA was extracted by a DNA extraction kit (SinaPure™ DNA, Sinaclon Co.). The sizes of *rhlA*, *rhlB*, and *rhlC* genes were 230, 301, and 436 bp, respectively. Also, the following primers were used in this research for multiplex PCR amplification of *P. aeruginosa* genes involved in rhamnolipid biosynthesis: for *rhlA* F: -ATCGGCCATCTGCTCAACGA- and R: -TCCTCGGCGGTGGTGTATTC-, for *rhlB* F: -TCTGCACCATCCCGGTGTTT- and R: -AAGGTCGATGGCGAGACCTG, and for *rhlC* F: -ATCTCGACGGACTGACGCTC- and R: -TTGAGCAGCTTGTCGCGTTC. Multiplex PCR reaction was performed in a final volume of 25 µL under the following thermal cycling program: a preliminary cycle involving a denaturation step for 3 min at 94 ˚C; followed by 35 cycles comprising denaturation at 94 ˚C for 60 s, annealing at 60 ˚C for 30 s, and extension at 72 ˚C for 60 s; and a final cycle

involving an extension step at 72 ˚C for 10 min. PCR products were evaluated using agarose gel (1%) electrophoresis run at 100 volts for 45 min and a 100 bp ladder (Sinaclon Co.). In this research, the standard strain *P. aeruginosa ATCC27853* with a strong biofilm formation ability was used as a positive control.

**Swarming assay**: In a previous study, swarming motility was studied on M8 agar (0.5%) containing glutamate (0.05%) and glucose (0.4 %) [12]. In another study, M8 minimal medium, complemented with casamino acids (CAA) (0.5%), glucose (0.2%), and $MgSO_4$ (1 mM) and solidified with agar (0.5%), was used as a swarming motility agar [13]. In this study, Bushnell Haas agar (0.5%) was used as a motility medium because this medium contains all the necessary minerals, except carbohydrates. Bushnell Haas medium (Himedia Co.) was composed of 0.05 g/L ferric chloride ($FeCl_3$), 1.00 g/L ammonium nitrate ($NH_4NO_3$), 1.00 g/L dipotassium phosphate ($K_2HPO_4$), 1.00 g/L monopotassium phosphate ($KH_2PO_4$), 0.02 g/L calcium chloride ($CaCl_2$), and 0.2 g/L magnesium sulfate ($MgSO_4$), the pH was adjusted to 7.0, and 2% glycerol was used as the sole carbon source [14]. The center of Bushnell Haas agar plates was spotted by 3 µL of LB (lysogeny broth) containing bacterial suspension with an OD600 equivalent to 3 McFarland standard. The plates were then subjected to incubation at 37 ˚C for 24 and 72 hrs.

## Findings

Among the 68 *P. aeruginosa* strains, 100% of the isolates were biofilm producers, 21 (30.9%) strains were strong biofilm producers, 25 (36.8%) strains were moderate biofilm producers, and 22 (32.4%) strains were weak biofilm producers (Fig. 2). They had a normal distribution.

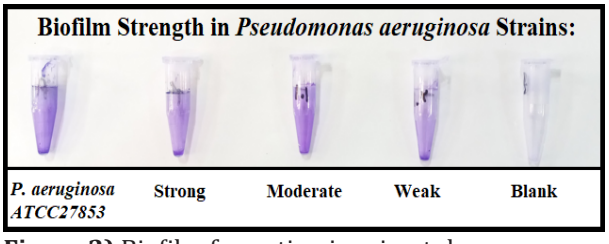


**Figure 2)** Biofilm formation in microtubes

**Rhamnolipid genes:** The frequency of *rhlC* among *P. aeruginosa* strains with strong, moderate, and weak biofilm formation ability was 81, 92, and 86.4%, respectively; the frequency of *rhlA* and *rhlB* among both strong and moderate biofilm-producing isolates was 100%, while this value was 95.5% for weak biofilm producers (Table 1). The frequency of *rhlC* was significantly different from those of *rhlA* and *rhlB* ($p$= .01). Despite biofilm-stabilizing effects of rhamnolipids, they had no significant effect on biofilm strength ($p$= .5). DNA fragments of *rhlA, rhlB,* and *rhlC* were placed in the expected sites on the gel at 230, 310, and 436 bp, respectively (Fig. 3).

**Colonies and velocity:** *P. aeruginosa* strains cultured on Bushnell Haas agar (0.5% agar) with two carbon sources (glycerol and glucose) were assessed after 24 and 72 hours. The pattern of bacterial colonies and movement was different based on bacterial strain and type of carbon source. Bacterial colonies with different strengths in biofilm

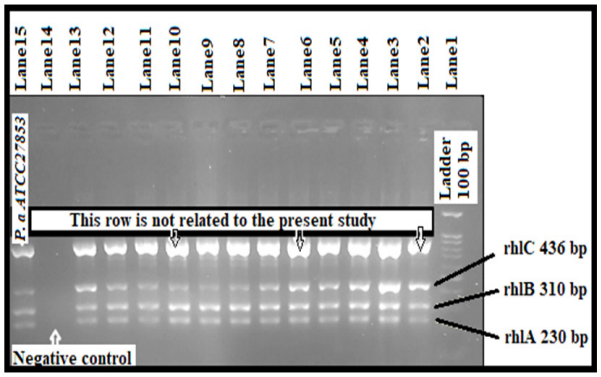


**Figure 3**) Multiplex PCR amplification of *P. aeruginosa* genes involved in rhamnolipid biosynthesis (*rhlA*, *rhlB*, and *rhlC*): lane 1: DNA ladder (100 bp), lane 2-13: clinical samples of *P. aeruginosa*, lane 14: negative control, and lane 15: positive control; the bands of *rhlA*, *rhlB*, and *rhlC* are 230, 310, and 230 bp, respectively.

formation in glucose-containing media had two parts: the peripheral part seemed to be more active in swarming motility, and the central part showed less motility. But in glycerol-containing media, bacterial strains with different strengths in biofilm formation showed uniform colonies: there was no significant difference between the peripheral and central parts of colonies (Fig. 4 and Fig. 5).

Bacterial velocity on Bushnell Haas agar (0.5% agar) with glucose as the sole carbon source was 2 µm/min in the first 24 hours, which decreased after 72 hours from 2 to 1 µm/min for all strains (Fig. 4). In addition, bacterial velocity on Bushnell Haas agar (0.5% agar) with glycerol as the sole carbon source was 2 µm/min in the first 24 hours. After 72 hours, it decreased by 50% for strong biofilm producers but remained constant for strains with moderate and weak biofilms (Fig. 5). It means that the velocity of strong biofilm producers decreases two times compared to both moderate and weak biofilm producers ($p$= .02).

## Discussion

*P. aeruginosa* is recognized as a causative agent of various infectious diseases associated with high morbidity and mortality rates, especially among immunocompromised individuals. Therefore, it is an alarm, and the factor or factors involved in this phenomenon should be identified, like resistance genes,

**Table 1)** Frequency distribution of *P. aeruginosa* genes involved in rhamnolipid biosynthesis (*rhlA*, *rhlB,* and *rhlC*) based on biofilm strength

| Biofilm Strength | N (%) | Rhamnolipid Genes | | |
|---|---|---|---|---|
| | | ***rhlA*** **, N(%)** | ***rhlB*** **, N (%)** | ***rhlC*** **, N (%)** |
| Strong | 21 (30.9) | 21 (100) | 21 (100) | 17 (81) |
| Moderate | 25 (36.8) | 25 (100) | 25 (100) | 23 (92) |
| Weak | 22 (32.3) | 21 (95.5) | 21 (95.5) | 19 (86.4) |

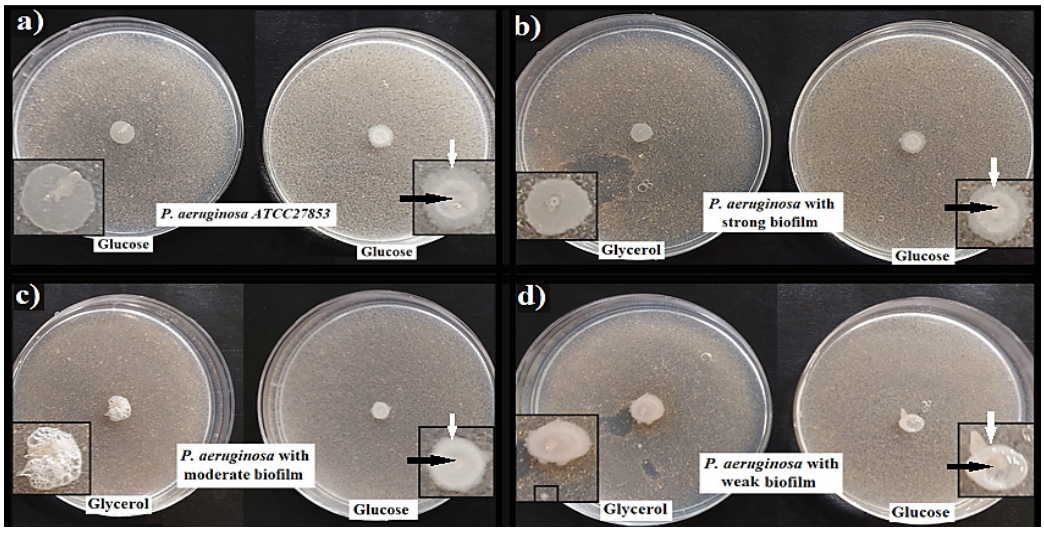


**Figure 4**) *P. aeruginosa* colonies on Bushnell Haas agar (0.5%) after 24 hours: a) *P. aeruginosa ATCC27853* with strong biofilm as a standard, b) isolated *P. aeruginosa* strains with strong biofilm, c) isolated *P. aeruginosa* strains with moderate biofilm, and d) isolated *P. aeruginosa* strains with weak biofilm. Black arrow: colony center with the least swarming motility, white arrow: colony peripheral with the greatest swarming motility.

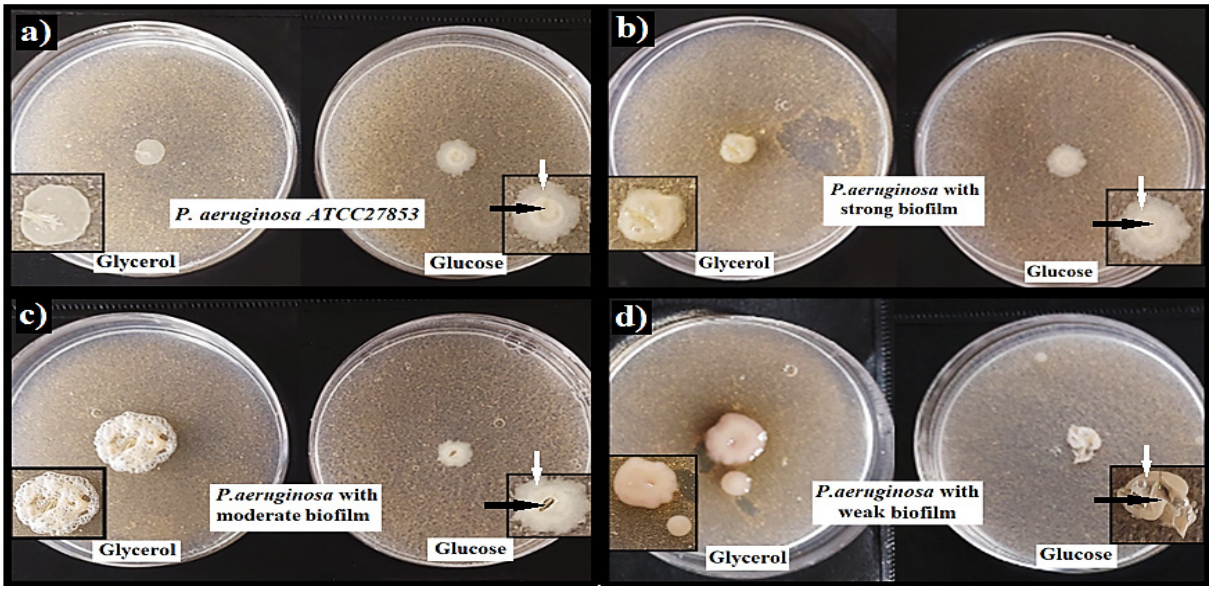


**Figure 5)** *P. aeruginosa* colonies on Bushnell Haas agar (0.5%) after 72 hours: a) *P. aeruginosa ATCC27853* with strong biofilm as a standard, b) isolated *P. aeruginosa* strains with strong biofilm, c) isolated *P. aeruginosa* strains with moderate biofilm, and d) isolated *P. aeruginosa* strains with weak biofilm. Black arrow: colony center with the least swarming motility, white arrow: colony peripheral with the greatest swarming motility.

transfer routes of these genes, virulence factors, and other factors participating in the spread or stability of *P. aeruginosa* biofilm. In this study, all 68 strains produced biofilm, and the frequency of strong, moderate, and weak biofilm producers was 30.9, 36.8, and 32.4%, respectively. In a meta-analysis study by Mirzahosseini et al. (2020) on the biofilm formation ability of *P. aeruginosa*, the combined frequencies of strong, moderate, and weak biofilm producers were 51, 29.2, and 25.4%, respectively [15]. In another study by Yousefpour and colleagues (2021), 100% of *P. aeruginosa* strains were biofilm producers, with near 27, 56, and 17% of them being strong, moderate, and weak biofilm producers [16]. Yakout and Abdelwahab (2022) showed that the frequency of strong, moderate, and weak biofilm producers was 53.5, 23.2, and 16.3%, respectively, while 7% were non-biofilm producers [17]. In a study by Shatti and colleagues (2022), 47.5, 30, and 22.5% of biofilm-producing strains formed strong, moderate, and weak biofilms, respectively [18]. Davarzani et al. (2021) showed that 14 (45%) isolates were strong, 12 (38%) isolates were moderate, and 5 (16%) isolates were weak biofilm producers [19]. One of the reasons for the difference in the results of various studies is related to the sampling sites; for example, *P. aeruginosa* strains isolated from urine and wound samples produce stronger biofilms compared to other clinical samples [19, 20]. In the present study, the frequency of *rhlA* and *rhlB* among both strong and moderate biofilm-producing isolates was 100%, while this value was 95.5% for weak biofilm producers. The frequency of *rhlC* among strong, moderate, and weak biofilm producers was 81, 92, and 86.4%, respectively; also, the distribution pattern of *rhlC* among these three groups was normal. We found no reports on the frequency of *rhlA, rhlB*, and *rhlC* in the literature. In this study, rhamnolipids in *P. aeruginosa* strains played a significant role in biofilm formation ($p< .05$). According to the results, *rhlA* and *rhlB* had no significant effect on biofilm strength ($p= .99$), but *rhlc* played a significant role in biofilm strength ($p= .03$). Three of the most important biosurfactants in *P. aeruginosa* are as follows: L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-

hydroxydecanoate (di-rhamnolipid), L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (mono-rhamnolipid), and 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs) [21]. RhlA enzyme is responsible for the production of HAAs, and RhlB enzyme is responsible for converting them into mono-rhamnolipids. Finally, Rhl-C enzyme changes mono-rhamnolipids to di-rhamnolipids [22]. Quorum-sensing (QS) systems vigorously regulate the rhlAB operon, and the synthesis of rhamnolipids usually takes place during the growth stationary phase [23]. Studies have shown that at low rhamnolipid concentrations, lipopolysaccharide (LPS) release and surface hydrophobicity of *P. aeruginosa* increase [7, 24]. Subsequently, the surface adhesion of bacteria increases as a necessary condition for the initiation of microcolony formation in biofilms [25]. Rhamnolipids contribute to the creation and maintenance of biofilm fluid channels for the circulation of water and oxygen in biofilm structure, especially in the base of the biofilm [26]. A study by Pamp and Tolker-Nielsen (2007) showed that the mushroom cap of *P. aeruginosa* biofilm could be produced by biosurfactants [25]. In general, studies have confirmed the role of rhamnolipids as a biosurfactant in biofilm formation; but no study was found to show the role and relationship of *rhlA, rhlB*, and *rhlC* genes

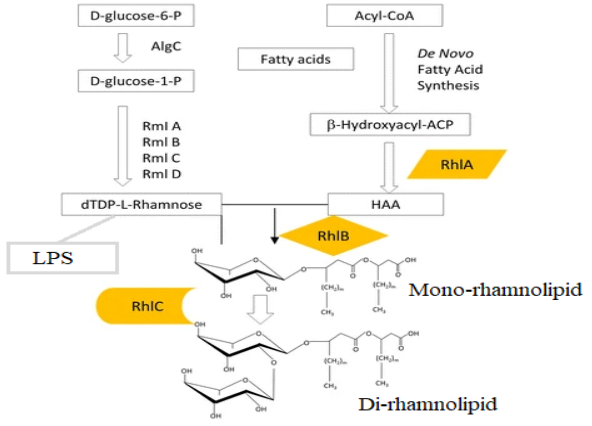


**Figure 6)** Rhamnolipid and LPS synthesis in *P. aeruginosa* [7]

with biofilm strength of *P. aeruginosa*.

In this study, after 72 hours of incubation on Bushnell Haas agar with glycerol and glucose, strong biofilm-producing strains of *P. aeruginosa* showed a 50% decrease in swarming motility velocity, but moderate and weak biofilm-producing strains showed no significant difference in velocity on Bushnell Haas agar (0.5% agar) with glycerol. Swarmer cells are translocated rapidly along their axis in multicellular groups, these cells are hyperflagellated and elongated. Also, exopolysaccharides as a slime layer surround them [27]. A reason for the linkage between swarming motility and biofilms in *P. aeruginosa* is the production of rhamnolipids which themselves are involved in biofilm development. In the first step, the release of lipopolysaccharides and the increase of cell surface hydrophobicity are induced by rhamnolipids. This helps planktonic cells attach to surfaces [27]. In general, biosurfactants (bioemulsifiers) play a crucial role in controlling the attachment or detachment of microbial cells from surfaces [28]. Additionally, emulsifiers play an important role in bacterial biofilm formation, quorum sensing, and pathogenesis. In biofilm formation, rhamnolipids alter cell-cell and cell-surface interactions [29].

In a study by Christova et al. (2011), among the four carbon sources, n-hexadecane and glycerol were better for the production of biosurfactants as surface tension reducers [30]. In addition, Moussa and colleagues (2014) showed that rhamnolipid yield from glucose and glycerol was 0.3±0.01 and 0.25±0.02, respectively. Also, surface tension for glucose and glycerol was 34±1.2 and 46±0.99 (dyne/cm), respectively [31]. These results confirm the present study findings regarding the swarming motility on Bushnell Haas with glucose compared to glycerol; moderate and weak biofilm producers could move easily with better velocity on glycerol-

containing media compared to glucose-containing media. In this study, rhamnolipids produced from different carbon sources showed different behaviors on colony shape, velocity, and strength of bacterial biofilms.

## Conclusion

Investigating the ability of *P. aeruginosa* to produce rhamnolipids seems very important, considering that there are different combinations of carbohydrates in different tissues of this bacterium, which could cause different behaviors in rhamnolipid production and thus change the movement pattern, the spread of bacteria in tissues, the formation of biofilms with different strengths, and the appearance of other pathogenic agents.

**Abbreviations:** rhl (rhamnolipid), *P. aeruginosa* (*Pseudomonas aeruginosa*), C4-HSL (N-butanoyl-L-homoserine lactone), PCR (polymerase chain reaction), BHI broth (brain heart infusion broth), ELISA (enzyme-linked immunosorbent assay), $OD_C$ (optical density of control), HAA (3-[3-hydroxyalkanoyloxy] alkanoic acids)

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**Authors Contribution:** SM and AT conceived and designed the experiments. SM and SA performed the experiments. AA analyzed the data. AT wrote the manuscript. MAH helped in editing the text of the article. All authors reviewed and approved the manuscript.

**Conflicts of interests:** The authors of this study declare that there is on conflict of interest.

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**Consent to participate:** Written consent was obtained from the participants.

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