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Antagonistic, biofilm-forming rhizospheric *Pseudomonas* spp. isolated from Hail province

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Abstract

Background: The objectives of this study were to characterize *Pseudomonas* rhizospheric strains, that have a biocontrol activity, in rhizosphere soil in Hail province and study their ability to form biofilm.

Methods: Rhizosphere soil samples were collected from rhizosphere of soil plantation areas, to be used for bacteria isolation. The identified bacteria were qualitatively tested for their ability to produce slime and subsequently develop biofilm.

Results: The cultural and biochemical identification techniques, including morphological, biochemical and molecular methods revealed that the antagonistic bacteria- from the distinctive rhizosphere soil samples belong to *Pseudomonas* genus in particular, *P. aeruginosa* (PF1a, pf2a, PF-8) and *P. putida* (PF-7). These identified isolates inhibited *Aspergillus niger* development with percentage of parasitic growth inhibition greater than (48.095 ± 2.182)% for *P. aeruginosa* (pf-8). In addition, these identified isolates were significantly shown to be able to produce exopolysaccharide and subsequently develop biofilm on polystyrene and glass surfaces.

Conclusion: Superior strains of these bio-control and plant growth promoting rhizobacteria will enable for better biological control of fungal and bacterial plant diseases and may reduce chemical pesticide usage. The indigenous strains isolated could potentially have a great impact on controlling plant diseases, in particular, those caused by microorganisms, and could be used as an alternative bio control agents instead of harmful chemical pesticides. Most of the tried microbes produced exopolysaccharides as well as formed biofilm on polystyrene and glass surfaces.

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Introduction

Plant diseases are with human beings since agriculture began. The population explosion has caused a desire for a lot of food with increasing quantity of land dedicated to crop production. Intensification and mono cropping have resulted in increasing sickness pressure. Although the artificial fertilizers and pesticides turn out high crop yields, environmental balances are non-continuous and tidy crop harm by insects and pathogens still prevail. Plant diseases contribute 13-20% of losses in crop production worldwide [1]. Some fungi and bacterium present in soils and rhizosphere zone of numerous crops function a possible biocontrol agent against various plant pathogens. Fungi within the genus *Trichoderma* and bacterium within the genera of Pseudomonas and Bacillus are of increasing interest as bioprotectants against plant diseases [2].

There has been associate enlarged intrigued in natural management operators in past decades [3]. A lot of range of biocontrol operators were examined for their efficaciousness and ecological influence as well as mammalian security. Several organisms are examined as potential agents for the vector dipterous insect management, as well as many other organism. However, most of those operators were not effective [4]. Pathogenic microorganisms deliberate or weaken their host and comparatively they are host-specific. Numerous microorganism insect diseases infections happen normally, however, could be utilized as biological pesticides. When actually happening, these flare-ups are density-dependent in this they - ordinarily as it were happen as insects ended up denser [3].

Pseudomonadaceae may be a massive and vital family of Gram-negative bacteria, including the genera *Pseudomonas*, genus *Xanthomas*, *Zoogloea* and *Frateuria* [1]. The *Pseudomonas* genus is especially fascinating, because of some strains are vital not solely medically, however, additionally vital environmentally and agriculturally, some are phytopathogens and lots of others are plant growth promoters. These microorganisms are principally found in soil and water [5, 6]. The *Pseudomonas* genus has been heterogeneous since Migula to begin with it in 1984 [7]. He selected and delineated the species related to the genus in 1895. Individuals of the bacteria have been described by metabolic skillfulness, one or numerous flagella as well as high (G+C) content [8, 9]. Identification of superior strains of the biocontrol rhizobacteria can alter higher biological management of serious plant diseases and should cut back chemical usage. Therefore, this study aimed to identify *Pseudomonas* bacteria, which has a biocontrol activity in rhizosphere soil in Hail province as well as the biochemical characterization of biofilm-forming rhizospheric *Pseudomonas* spp., from Hail agricultural soil.

Methods

Study area

The study was conducted at Biology Department, College of Science from November 2018-December 2019) in Hail province, KSA.

Soil sampling and isolation of *Pseudomonas*

Soil samples were collected from rhizosphere of soil plantation areas in which olive, orange, grape (fruit tree), Annual Rye Grass (*Lolium multiflorum*) (ornament seedling) and potato (vegetable) cultivated in the nursery of Ministry of Agriculture (Al-Gayed-North of Hail region, Saudi Arabia). Soil samples were taken from 3-5 cm depth of irrigated fields and then placed in sterile containers, to be taken to the laboratory. Five grams of root and closely adhering soil was suspended in a flask containing 50 ml of sterile distilled water. Flasks where were placed on a rotary shaker at 200 rpm for 10 minutes. For isolation of the bacteria, standard spread-plate dilution method was conducted[8] in triplicate. Serial dilutions (tenfold) of the mixture were plated on CHROMID *Pseudomonas aeruginosa* agar (Biomerieux, France). After incubation for 24 hours at 37°C, visible discrete blue-green colonies in incubated plates were purified on nutrient agar and prepared for further analysis.

Morphological and biochemical tests of the bacteria

The isolates were hypothetically identified by implies of morphological examination and a few biochemical characterizations including: morphology of colonies, Gram staining, protease production, cellulase production, catalase generation, Voges Proskauer reaction, indole generation, starch hydrolysis, citrate utilization, and gelatin hydrolysis. The results were compared with Bergey's Manual for Determinative Bacteria [10-12]. Biochemical fermentation test was utilized to determine whether bacteria could ferment glucose, galactose and lactose in peptone broth medium [13].

Identification of the bacterial strains based on 16S rRNA gene sequence analysis

The identification of the bacterial isolates based on 16S rRNA gene sequence analysis was accomplished by 16s rRNA Packaged service through Sanger sequencing as offered by Macrogen Inc., (Korea). This packaged service includes gDNA extraction, PCR amplification, PCR product purification, and bidirectional sequencing, and universal primers were used. In these methods**,** Genomic DNA was extracted from the bacterial isolates and used as template in a 30-µL reaction mixture by using an EF-Taq DNA polymerase (SolGent, South Korea).

Polymerase Chain Reaction (PCR)

Amplification of the 16S rRNA gene from bacterial isolates was carried out using the universal primers; 27F 5′-AGA GTT TGA TCM TGG CTC AG-3′ and 1492R 5′-TACGGYTACCTTGTTACGACTT [14], in a total 50 µl of PCR reaction. The PCR cycle consist of three main steps: denaturation at 94°C for 45s, annealing at 55°C for 60s, and extension 72°C for 60 s. The PCR program was run in 30 amplification cycles, followed by a final extension at 72°C for 10 min. The obtained PRC

products were purified using QIAquick® PCR Purification Kit (Cat. No. 28106) according to manufacturing procedures. Macrogen Inc., (Korea), sequenced the purified PCR products and sequencing of the purified isolates was performed in both directions using 27F and 1492R primer pairs. Sequence alignments were edited by MEGA7 [15].

Sequencing and phylogenetic tree: The purified PCR amplicons were sequenced by using two primers (27F 5′-AGA GTT TGA TCM TGG CTC AG-3′ and 1492R 5′-TACGGYTACCTTGTTACGACTT). Sequencing were performed by using Big Dye Terminator Cycle sequencing kit v.3.1 (Applied BioSystems, USA). Sequencing products were resolved on Applied BioSystems model 3730 XL automated DNA Sequencing System at Macrogen, Inc., Seoul, Korea. The primers 785F 5' (GGA TTA GAT ACC CTG GTA) 3' and 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3' are used for the sequencing of 16 rRNA.

The resulting nucleotide sequence measuring 997 bp in size was submitted to the GenBank sequence database (accession number MF425602.1). The sequence obtained were analysed using Basic Local Alignment Search Tool (BLASTN) and matched against known bacterial sequences present in NCBI GenBank data base [16]. Related sequences obtained from the database were used to construct a phylogenetic tree using MEGA5 software [17].

The Clustal X program [18] was used for multiple sequence alignments. Phylogenetic trees were constructed by the maximum-likelihood method based on the Jukes–Cantor model [19] using MEGA version 6 [17].

Antagonistic activity of the identified isolates: The ability of the identified Pseudomonas spp. strains to inhibit the growth of *A. niger* strain was tested by using the dual inoculation technique [20]. Nutrient agar Petri dishes plates were inoculated with the fungal suspension (10^6 spores/ml) and spot inoculated with each bacterial suspension (10^7 CFU/ml). Antagonistic activities were estimated by calculating the percentage of inhibition according the following equation (Eq.1):

Parentage inhibition = (D- T/ D) x 100

Where, (D) is the diameter of the *A. niger* colony in the control plate incubated for 96h; and (T) is the diameter of the fungus colony in the tested plates at the same period.

Qualitative and quantitative estimation of biofilm formation: The ability of the rhizospheric *Pseudomonas* strains was tested both qualitatively and quantitatively by using two techniques: the Congo red Agar and the Cristal violet technique. Congo red agar (CRA) method was tested using the method of Freeman *et al*. [21] to study the production of exopolysaccharides (slime) by the identified *Pseudomonas* spp. strains. For the experiment, nutrient agar medium containing 0.8 g/L of Congo red and 36 g/L of glucose was prepared. Petri dishes were incubated at 37°C for 24 h under aerobic conditions and followed by incubation at room temperature in dark for 24h. Pigmented colonies were interpreted as slime positive, whereas unpigmented bacteria were considered as slime-negative strains [22]. The ability of the *Pseudomonas* strains to form a biofilm on polystyrene 96-wells plate was quantified using the protocol [23]. *Pseudomonas* spp. strains were enriched in Brain Infusion Broth (BHI) then diluted (1:20 v:v) in new BHI medium supplemented with glucose (0.25% w/v). A volume of 200 µl of each bacterial suspension was used to inoculate sterile 96-well polystyrene microtiter plates (Fisher scientific, Sweden). After incubation at 37°C for 24h, the cultures were removed and the wells were washed twice and air-dried in an inverted position. Crystal violet solution (1%, w/v) was used to stain the adherent bacterial cells for 15mn. 200 µl of ethanol–acetone (80:20 v/v) was used to solubilize the dye used. The optical density (OD_{595}) was measured spectrophotometerically (Bio TeK Instruments, USA). Each essay was performed in triplicate.

A 24-well microtiter plates (Fisher scientific, Sweden) were used to test the ability of the identified *Pseudomonas* spp. strains to form a biofilm on glass surface 2ml of an overnight culture suspension was added to each well to allow bacterial adhesion after 24h of incubation at 37°C in shaker at 150 rpm Crystal violet (1%, w/v) solution was used to stain the adherent cell for 5 mn. Acetic acid 33% (w/v) was used to dissolve the satin and the optical density was recorded at 570nm using a microtiter plate reader (Bio TeK Instruments, USA). Negative controls were obtained by placing the materials in PBS without bacterial cells. The experiments were performed in triplicate. We used the scheme proposed by Stepanović *et al*. [24] to interpret the obtained results as follow: non-adherent (0): OD≤ODc; weakly adherent (+): ODc<OD≤2×ODc; moderately adherent (++): 2×ODc<OD≤4×ODc; strongly adherent (+++): 4×ODc<OD.

Results

Cultural and morphological features of the isolated strains: Twenty samples were harvested from rhizosphere of soil plantation areas in which olive, orange, grape (fruit tree), Annual Rye Grass (*Lolium multiflorum*; ornament seedling) and potato (vegetable) from Hail region, Saudi Arabia). On the CHROM^{ID} *Pseudomonas aeruginosa* agar, blue-green colonies were detected (Figure 1).

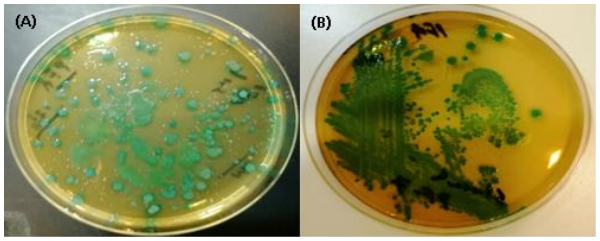

Figure 1: Different morphotypes of rhizospheric bacteria isolated on CHROMID Pseudomonas aeruginosa agar (A), purified Blue-green colonies of PF7 strain (B).

All the isolates showed, smooth shiny surface. Based on the morphological and cultural characteristics of the isolates on the KB medium and observation of pigmentation under UV light about thirty colonies from above plates were selected, purified and the pure cultures obtained was stored in refrigerator at 4°C (Table 1).

Biochemical characterization of the bacterial isolates

Results of this analysis are presented in Table (2). In fact, all tested isolates were negative for indole production, three isolates for methyl red test, seven isolates for Voges Proskauer's test showed positive results. All the bacterial isolates were positive for gelatin liquefaction. All the isolates were citrate, catalase and oxidase positive strains. The tested bacteria produced several exoenzymes including gelatinase. Eight isolates showed positive results for cellulose production. Additionally, glucose, galactose and lactose sugars supplemented in liquid media were utilized by seven isolates, six isolates and eight isolates, respectively. i.e., the tested bacteria were able to utilize the carbohydrates and these tests confirmed the strains biochemically as *Pseudomonas* members.

Identification of bacteria by 16S rRNA gene sequencing: The obtained sequences were edited in MEGA7 and compared to available sequences in the GenBank database. 16S rDNA sequencing showed that bacteria isolated from the various rhizosphere of soil plantation samples were mostly belonged to *Pseudomonas* genus. Two species were identified *P. aeruginosa* and *P. putida* (Figure 2). Three strains belonging to the species *P. aeruginosa* was identified namely pf1a (accession number: MN759721-MN759722), pf2a (accession number: MN759723-MN759724) and pf-8 (accession number: MN759727-MN759728). The strain pf-7 was identified as *P. putida* species (accession number: MN759725-MN759726). The strain pf9f was identified as member of *Pseudomonas* genus (accession number: MN759729).

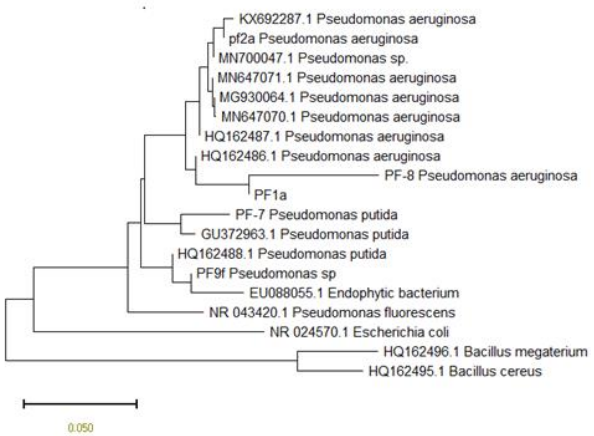

Figure 2: Phylogenetic tree using the Neighbor-Joining method of the five rhizospheric *Pseudomonas* spp. strains (PF1a, PF9f, PF-8, pf2a, and PF-7) as compared to some close-related reference strains based on 16S rRNA gene sequence.

Fungi-toxic potential of the obtained *Pseudomonas* spp. strains: The obtained results (Figure 3) showed that three strains tested showed a high degree of growth inhibition tested on *A. niger* strain with a percentage about (35.238±0.824)% for *P. aeruginosa* (pf1a), (48.095±2.182)% for *P. aeruginosa* (pf-8), and (42.857±1.65)% for *P. aeruginosa* (pf2a). No fungal growth inhibition was recorded when *Pseudomonas* sp. (pf9) and *P. putida* (pf-7) were tested.

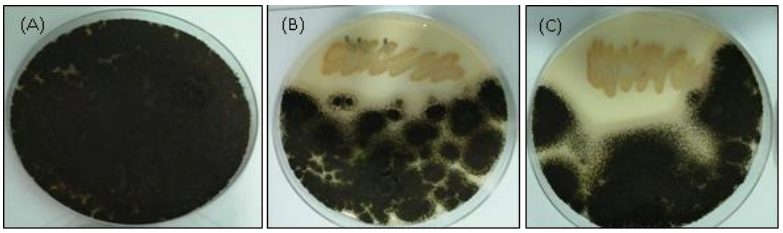

Figure 3: Fungi-toxic activity of the selected bacterial strains against *A. niger* strain. (A): *A. niger* without treatment, (B): *P. aeruginosa* (pf1a) strain and (C): *P. aeruginosa* (pf-8) strain.

Slime production and biofilm formation of the bacterial isolates: Phenotypic production of exopolysaccharides was tested by streaking the identified *Pseudomonas* spp. strains on Congo Red agar Petri dish plates. Among five strains, two were slime producer strains giving a black colonies (Figure 4). Three strains are non-producer-strains with a pink-red morphotype. All *Pseudomonas* strains were screened for their adherence to polystyrene (96 well microtiter plates) and glass surfaces (Figure 5) by using the Cristal Violet technique. The obtained results showed that all strains formed a biofilm on the two surfaces with different degree (Table 3). Four strains are classified as medium biofilm bacteria (2×ODc<OD≤4×ODc). All tested strains adhered to glass surface except the strain PF8 (strongly adherent strain).

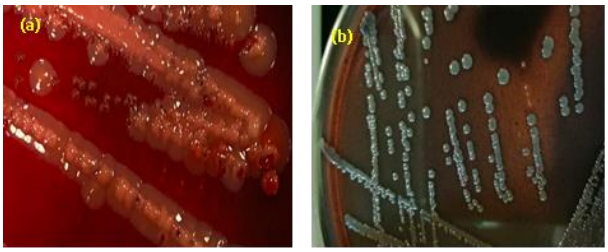

Figure 4: Different colorimetric scale morphotypes on Congo red agar. (a): unpigmented colonies (slime negative), (b) pigmented colonies 9slime positive).

Discussion

The bacterial isolates were identified and characterized using conventional methods as well as molecular biological techniques. Moreover, the antifungal action was tested, their slime production and biofilm formation were also investigated. The typical phenotypic tests are frequently used in microorganism identification.

Code	Size	Margin	Color	Elevation	Surface	Pigmentation	Gram reaction	Shape
pf1a	Small	Round	Yellowish green	Convex	Smooth shiny	Yellowish green	Negative	Rods
pf2a	Medium	Round	Dull white	Convex	Smooth shiny	Bluish green	Negative	Rods
pf3	Small	Round	Bluish white	Convex	Smooth shiny	Bluish green	Negative	Rods
pf4a	Small	Round	Yellow	Convex	Smooth shiny	Yellowish green	Negative	Rods
pf5a	Small	Round	Yellowish green	Convex	Smooth shiny	Dark green	Negative	Rods
pf6a	Small	Irregular	Yellowish green	Convex	Smooth shiny	Light green	Negative	Rods
pf7	Small	Irregular	Green	Convex	Smooth shiny	Dark green	Negative	Rods
pf8	Medium	Round	White	Convex	Smooth shiny	Light green	Negative	Rods
pf9f	Small	Irregular	White	Convex	Smooth shiny	Light green	Negative	Rods
pf10	Small	Round	Bluish white	Convex	Smooth shiny	Light green	Negative	Rods

Table 1: Cultural and morphological features of the selected isolates.

No.	Indole test	MR test	VP test	C.U	Catalase	Oxidase	P.P	G.L.	C.L.	Carbohydrate utilization		
										Glucose	Galactose	Lactose
1	-	+	+	+	+	+	-	+	+	-	+	+
2	-	-	+	+	+	+	-	+	+	+	+	-
3	-	+	-	+	+	+	-	+	+	+	+	+
4	-	-	+	+	+	+	+	+	+	-	+	+
5	-	+	+	+	+	+	-	+	-	+	-	+
6	-	+	-	+	+	+	+	+	+	+	-	+
7	-	-	-	+	+	+	-	+	+	+	+	+
8	-	+	-	+	+	+	+	+	-	+	-	-
9	-	+	-	+	+	+	+	+	+	+	-	+
10	-	+	+	+	+	+	-	+	+	-	+	+

C.U.: Citrate utilization P.P.: Protease production; GL: Gelatin liquefaction; C.L: Cellulase production

Table 2. Biochemical properties of the presumptive *Pseudomonas* spp. strains

Strain/Origin	Qualitative Assay		Qualitative Assay	
	Morphotype on Congo Red Agar	Slime Production	Polystyrene (OD_{595} ± SD)*	Glass (OD_{595} ± SD)**
PF1	Pink-red	Non Producer	+	M
PF2A	Black	Producer	++	M
PF7	Pink-red	Non Producer	++	M
PF8	Pink-red	Non Producer	++	S
PF9	Black	Producer	++	M

*OD595: (-) non-biofilm forming OD595 ≤ 1; (+) weak biofilm forming 1 < OD595 ≤ 2; (++) medium biofilm forming 2 < OD595≤ 3; (+++) strong biofilm forming OD595≥ 3

**Adhesion to glass material was interpreted according to the scheme of Stepanovic *et al.*' [24].

Table 3. Qualitative (Congo Red Agar) and quantitative (Cristal Violet technique) estimation of biofilm formation by the identified *Pseudomonas* spp. Strains.

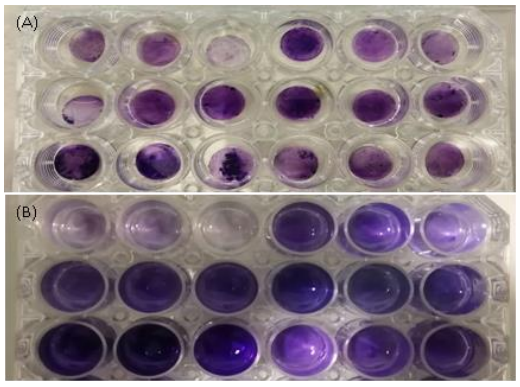

Figure 5: Adhesion of *Pseudomonas* spp. strains to glass biomaterial. (A): Glass slides after coloration with crystal violet, (B): After addition of acetic acid 33%.

The classical phenotypic traits include morphological, physiological, and biochemical features [25]. In the present study, the bacterial preliminary identification tests revealed that all bacterial isolates belong to *Pseudomonas* genus. Similar results were obtained by Jayashree *et al*., [26]. A variation in the colony colour might be ascribed to the generation of various colors metabolites [27]. These characteristics were taxonomically useful characteristics for Pseudomonas [28-30].

The biochemical characterization of the bacterial isolates revealed their belonging to *Pseudomonas* spp. These results agreed with those of Sonia *et al*., [31], Magnelli and Forchiassin [32], Immanuel *et al*., [33]; Akter *et al*., [34]; Basha *et al*., [35] who isolated *Pseudomonas* spp. from pigeon pea rhizospheric soil and identified them using morphological and biochemical characterization. The 16srRNA gene sequence is a very commonly utilized system for the identification of bacterial species [36]. This technique comprises exceptionally preserved nucleotide sequences, interspersed with variable areas that are genus- or species-specific. PCR primers focusing on the saved districts of rRNA enhance variable sequences of the rRNA gene [37]. The analysis showed that the isolated bacteria belonged to *Pseudomonas* genus. Two main species were identified as *P. aeruginosa* (PF1a, pf2a, PF-8) and *P. putida* (PF-7). They showed high sequence homology (99%) with *P. aeruginosa.* The present study indicate that rhizosphere soil is colonized by *P. aeruginosa*- as well as *P. putida.* Pseudomonads have numerous characteristics that make them fitting as biocontrol and growth-promoting operator38]. t. Moreover, pseudomonads are liable for the regular inhibition of certain soil borne pathogens [39].

Biofilm producer microorganisms are generally associated with nosocomial and recurrent infections. Biofilm defined, as a sticky exopolysaccharide matrix, is the essential virulence factor leading to biofilm-related infections. This process begins with attachment of bacteria to biotic and abiotic surfaces. After attachment, aggregation of bacteria is started by using cell-cell adhesion. A biofilm involves any syntrophic consortium of microorganisms in which cells stick to each different and frequently also to a surface [40, 41]. These adherent cells end up embedded within a slimy extracellular matrix that is composed of extracellular polymeric elements (EPS) [40, 41]. The cells within the biofilm produce the EPS components, which are usually a polymeric conglomeration of extracellular polysaccharides, proteins, lipids and DNA [42]. Because they have three-dimensional structure and represent a neighborhood life style for microorganisms, they have been metaphorically described as "cities for microbes".

Previous studies have reported the use of the Congo red agar technique to access the ability of clinical and environmental Pseudomonas spp. strains to produce slime [42-46]. In fact, Baniya *et al*., [47] studied the ability of 85 *P. aeruginosa* clinical isolates to produce biofilm on the basis of tube adherence and the Congo Red method. They reported that only 10% were slime producer. While, Rewatkar and Wadher [43] founded that 90% of *P. aeruginosa* clinical strains were strong biofilm forming bacteria with black colonies on CRA medium. The remaining strain gave pink colour colonies indicating non-biofilm production.

Authors' Contribution

All auhtors contributed equally in designed, conducting and reporting this research.

Competing Interest

All the authors declare that they have no competing interest that can affect the current study.

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