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Qualitative and Quantitative Analysis of Siderophore Production from *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa (*P. aeruginosa*) is known for its production of a diverse range of virulence factors to establish infections in the host. One such mechanism is the scavenging of iron through siderophore production. *P. aeruginosa* produces two different siderophores: pyochelin, which has lower iron-chelating affinity, and pyoverdine, which has higher iron-chelating affinity. This report demonstrates that pyoverdine can be directly quantified from bacterial supernatants, while pyochelin needs to be extracted from supernatants before quantification.

The primary method for qualitatively analyzing siderophore production is the Chrome Azurol Sulfonate (CAS) agar plate assay. In this assay, the release of CAS dye from the Fe^{3+}-Dye complex leads to a color change from blue to orange, indicating siderophore production. For the quantification of total siderophores, bacterial supernatants were mixed in equal proportions with CAS dye in a microtiter plate, followed by spectrophotometric analysis at 630 nm. Pyoverdine was directly quantified from the bacterial supernatant by mixing it in equal proportions with 50 mM Tris-HCl, followed by spectrophotometric analysis. A peak at 380 nm confirmed the presence of pyoverdine. As for Pyochelin, direct quantification from the bacterial supernatant was not possible, so it had to be extracted first. Subsequent spectrophotometric analysis revealed the presence of pyochelin, with a peak at 313 nm.

Introduction

Organisms require iron to perform various vital functions, such as electron transport and DNA replication[1]. *Pseudomonas aeruginosa*, a Gram-negative opportunistic pathogen, is known to possess a variety of virulence factors to establish infection in the host, among which one mechanism is siderophore formation[2]. During iron-depleting conditions, *P. aeruginosa* releases specialized molecules called siderophores, which quench iron from the surrounding environment. Siderophores chelate iron

extracellularly, and the resulting ferric-siderophore complex is actively transported back to the cell[3].

P. aeruginosa is known to produce two siderophores, pyoverdine and pyochelin. Pyoverdine is known to have a higher iron chelating affinity (1:1), whereas pyochelin is known to have a lesser iron chelating affinity (2:1)[4]. Pyochelin is also called a secondary siderophore because it has a lower iron chelating affinity[5]. The production and regulation of siderophores are actively controlled by Quorum Sensing (QS) systems in *P. aeruginosa*[6].

Besides iron quenching, siderophores are also involved in regulating virulence factors and play an active role in biofilm formation[7]. Siderophores serve additional crucial roles, including involvement in cell signaling, defense against oxidative stress, and facilitation of interactions between microbial communities[8]. Siderophores are typically categorized based on the specific functional groups through which they chelate iron. The three primary bidentate ligands in this classification are catecholate, hydroxamate, and α-hydroxycarboxylate[3]. Pyoverdines are hallmarks of fluorescent Pseudomonas species such as *P. aeruginosa* and *P. fluorescens*[5]. They consist of a mixed green fluorescent chromophore coupled to an oligopeptide containing 6-12 amino acids. Several non-ribosomal peptide synthetases (NRPs) are involved in their synthesis[9]. Four genes involved in pyoverdine production and regulation are pvdL, pvdI, pvdJ, and pvdD[10]. Pyoverdine is also responsible for infection and virulence in mammals[11]. *P. aeruginosa* is noted to produce pyochelin in moderate iron-limiting conditions, while pyoverdine is produced during severe iron-limiting environments[12]. Two operons involved in pyochelin production are pchDCBA and pchEFGHI[13]. It is noted that in the presence of pyocyanin, pyochelin (catecholate) induces oxidative damage and inflammation and generates hydroxyl radicals, which are harmful to host tissues[11].

The Chrome Azurol Sulfonate (CAS) assay is widely adopted due to its comprehensiveness, high sensitivity, and greater convenience compared to microbiological assays, which, although sensitive, can be overly specific[14]. The CAS assay can be conducted on agar surfaces or in a solution. It relies on the color change that occurs when the ferric ion transitions from its intense blue complex to orange. The CAS colorimetric assay quantifies the depletion of iron from a Fe-CAS-surfactant ternary complex. This particular complex, consisting of metal, organic dye, and surfactant, has a blue color and exhibits an absorption peak at 630 nm.

This report presents a method for the qualitative detection of siderophore production, where one can detect the production of siderophores on an agar plate. A method for the quantitative estimation of total siderophore production in a microtiter plate and the detection and quantitative analysis of two siderophores, pyoverdine and pyochelin, from *P. aeruginosa*, is also provided.

Protocol

All bacterial isolates of *P. aeruginosa* were obtained from medical microbiology laboratories from Vadodara and Jaipur, India. All selected clinical isolates were handled in Biosafety Cabinet (BSL2) and utmost care was taken while handling bacterial isolates during the experiments. The commercial details of all the reagents/solutions are provided in the **Table of Materials**.

1. Preparation of Chrome Azurol Sulfonate (CAS) dye and agar media

1. Prepare CAS dye (100 mL) with the following composition:
    1. Dissolve 60 mg of CAS in 50 mL of distilled water (Solution 1).
    2. Dissolve 2.7 mg of $FeCl_3 \cdot 6H_2O$ in 10 mL of 10 mM HCl (Solution 2).
    3. Dissolve 73 mg of Cetrimonium bromide (HDTMA) in 40 mL of distilled water (Solution 3).
    4. Carefully mix Solution 1, Solution 2, and Solution 3. Store it in a glass bottle.
2. Prepare CAS agar following the steps below:
    1. Add 100 mL of MM9 salt solution to 750 mL of distilled water.
    2. Dissolve 32.24 g of piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) free acid.
    3. Add 15 g of Agar agar. Autoclave and allow it to cool.
    4. Add 30 mL of sterile Casamino acid solution and 10 mL of sterile 20% glucose solution to the mixture.
    5. Add 100 mL of CAS dye, mix, and pour it in aseptic conditions.

        NOTE: Preparation of MM9 media, Casamino acid solution, and 8-Hydroxyquinoline are provided in **Supplementary File 1**. PIPES buffer is pH-sensitive. It will not dissolve until pH 5.6 is achieved. Ensure that the pH is constantly monitored because when PIPES starts to dissolve in water, it will further lower the pH. Extract Casamino acid with the same volume of 3% 8-hydroxyquinoline dissolved in chloroform. Leave the immiscible solution at 4 °C for around 20 min and carefully collect the upper phase of the solution without disturbing the lower phase.

2. Qualitative analysis of siderophores production

1. Set the OD_{600} nm to 0.2 for 24 h grown cultures of *P. aeruginosa*.
2. Using a sterile wire loop, streak the bacterial culture on a CAS agar plate.
3. Incubate at 30 °C for 24 h.

    NOTE: Peptone water media or 0.8% normal saline can be used to dilute the bacterial culture. If no bacterial growth is observed at 24 h, incubate CAS plates for 48 to 72 h.

3. Quantitative estimation of total siderophores

1. Re-inoculate 24-h-grown cultures of *P. aeruginosa* in Peptone water media after adjusting OD_{600} nm to 0.25, and incubate at 30 °C for 48 h.
2. After 48 h, centrifuge the bacterial culture at 4650 x *g* for 10 min at room temperature.
3. After centrifugation, add 100 µL of cell-free supernatant to a 96-well microtiter plate and add 100 µL of CAS dye to it.
4. Cover the plate with aluminum foil and incubate at room temperature for 20 min.
5. After incubation, take spectrophotometric readings at 630 nm.
6. Calculate the results obtained for the quantification of total siderophores as Percent Siderophore Unit (PSU).

    NOTE: PSU can be calculated as: $[(A_r - A_s)/A_r] \times 100$

    where, A_r = absorbance of the reference at 630 nm, A_s = absorbance of the cell-free supernatant of the

sample. For the reference, CAS dye should be added to uninoculated peptone water media. Fill all glassware, such as test tubes, flasks, etc., with 6 M HCl for 2 h and rinse them twice with distilled water to remove any trace of iron on them.

4. Quantitative estimation of pyoverdine

1. Re-inoculate 24-h-grown cultures of *P. aeruginosa* in Peptone water media after adjusting OD_{600} nm to 0.25 and incubate at 30 °C for 48 h.
2. After 48 h, measure the OD_{600} nm of the bacterial growth before proceeding further.
3. Centrifuge the bacterial culture at 4650 x *g* for 10 min at room temperature.
4. After centrifugation, add 100 µL of cell-free supernatant to a 96-well microtiter plate and add 100 µL of 50 mM Tris-HCl (pH 8.0) to it.
5. Take spectrophotometric readings at OD_{405} nm.

5. Pyochelin extraction and spectrophotometry

1. Re-inoculate 24-h-grown cultures of *P. aeruginosa* in King's B media (**Supplementary File 1**) after adjusting OD_{600} nm to 0.25 and incubate at 30 °C for 24 h.
2. After 24 h, take 100 mL of culture and centrifuge at 4650 x *g* for 10 min at room temperature.
3. After centrifugation, add 5 mL of 1 M citric acid to the supernatant. Extract twice with 50 mL of Ethyl acetate.
4. Filter the organic phase with magnesium sulfate through a syringe filter. Store the filtered organic phase at -20 °C.
5. Take spectrophotometric readings at 320 nm.
   NOTE: As pyochelin is a highly unstable compound at room temperature, perform the extraction process on ice. Use a 50 mL sterile syringe for filter separation. Place cotton at the tip of the syringe and add 1 gm of magnesium sulfate on it. Fix a sterile syringe filter at the tip of the syringe and collect the filtrate in a sterile tube.

Representative Results

Before quantification of siderophores from clinical isolates, a qualitative screening for siderophore production was carried out to ensure siderophores production. Qualitative detection of siderophores from clinical isolates was observed by streaking bacteria on CAS agar plates. Three clinical isolates, namely MR1, TL7, J3, along with PAO1 (the reference strain), were selected for the study. All three clinical isolates and PAO1 showed positive results for siderophore production, where a clear orange halo around the bacterial growth on the blue agar surface indicated positive siderophore production (**Figure 1**). The halo formation on the CAS agar (**Figure 1**) gave a rough estimation of the siderophore production by clinical isolates. Hence, quantitative estimation of siderophore production using CAS reagent and liquid media was performed.

Total siderophore quantification was performed directly from the cell-free supernatant. Post-incubation, a color change was observed where yellow color indicated the removal of CAS dye from the Fe-complex. Here, CAS dye with uninoculated growth media was used as control, which was taken for siderophores calculation. There was no significant difference in total siderophore production in isolates MR1 and TL7, while there was a significant total siderophore production in J3 compared to PAO1 (**Figure 2**).

Next, pyoverdine quantification was performed directly from cell-free supernatants. Pyoverdines are released in cell environments, so the cell-free supernatant was used for pyoverdine quantification. A UV range scan by the spectrophotometer was also performed, where the peak at 380 nm confirmed the presence of pyoverdine (**Figure 3A**). Spectrophotometric readings were taken at 405 nm, and results were interpreted as OD_{405}/OD_{600} nm. All isolates showed pyoverdine production, where isolates MR1 and J3 showed significantly lower pyoverdine compared to PAO1, while no significant difference was observed for TL7 (**Figure 3B**).

Pyochelin cannot be directly quantified from the cell-free supernatant. It was not possible to extract pyochelin from 1 mL of cell-free supernatant, so it was extracted from 100 mL of cell-free supernatant. It was acidified with 1 M citric acid. As pyochelin is a highly unstable compound, the extraction process was performed on ice. Pyochelin detection was performed by running a UV range scan from a range of 300 to 600 nm, where the peak at OD_{320} nm confirmed the presence of pyochelin (**Figure 4A**). Pyochelin quantification was performed by taking spectrophotometric readings at OD_{320} nm. Calculations were made by dividing OD_{320}/OD_{600}. Isolates MR1, TL7, and J3 showed significantly higher production of pyochelin compared to the reference isolate PAO1 (**Figure 4B**).

Comparative siderophore production in various bacterial growth media was performed to check the amount of total siderophores produced in various growth media. Four different media were selected, where Luria broth, King's B media, and Peptone water were extracted with 3% 8-hydroxyquinoline, and unextracted Luria broth was selected for the study. There was no significant difference between siderophore production in Extracted Luria broth, King's B media, and Peptone water media, while a significant difference was observed for siderophore production in unextracted Luria broth.

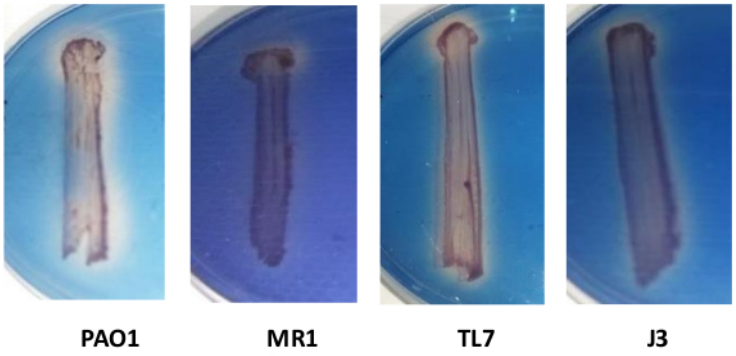

Figure 1: Siderophore production on CAS agar. Growth of *P. aeruginosa* on CAS agar plates of 24-h-grown cultures were spotted onto CAS agar plates and incubated at 30 °C for 24 h. Siderophore presence is indicated by an orange halo around the bacterial colony (PAO1, MR1, TL7, and J3). Please click here to view a larger version of this figure.

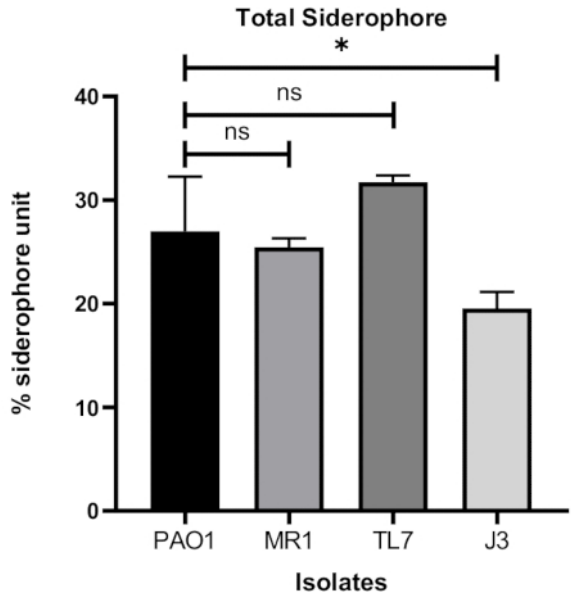

Figure 2: Total siderophores production. Total siderophore production was assessed for PAO1 and three clinical isolates (MR1, TL7, and J3). 100 µL of cell-free supernatant (of PAO1, MR1, TL7, and J3 grown for 24 h in peptone water broth) was added to a 96-well microtiter plate, thereafter 100 µL of CAS dye added to it. One-way ANOVA test was performed for statistical significance. Error bars represent ± standard deviation (SD) between three biological replicates. *corresponds to $p < 0.05$; ns corresponds to $p > 0.05$. Please click here to view a larger version of this figure.

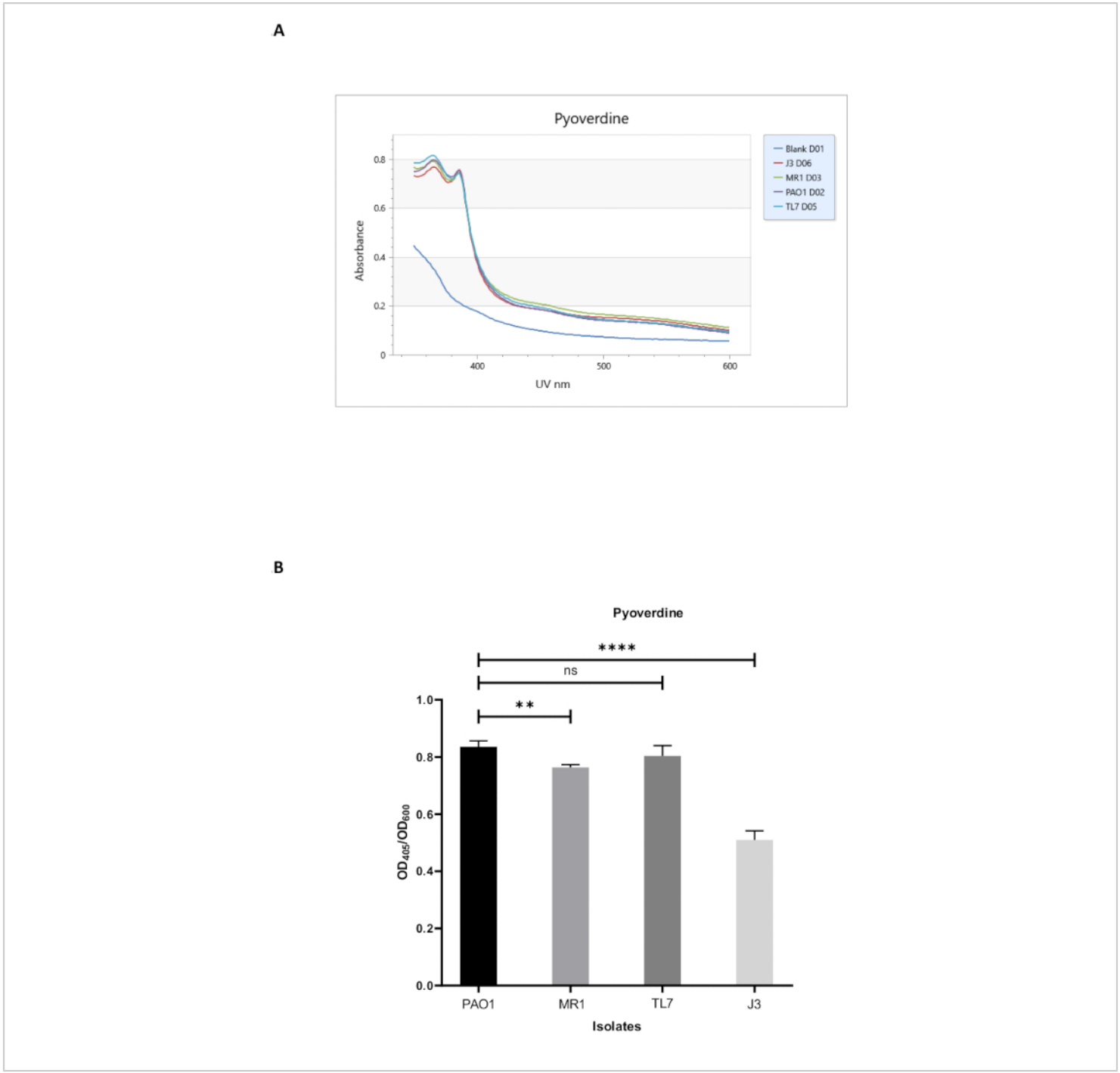

Figure 3: Pyoverdine production. Pyoverdine production of *P. aeruginosa* strain PAO1 and clinical isolates (MR1, TL7, and J3) assessed after 24 h of growth in peptone water media. The bacterial cultures were centrifuged, and 100 µL of cell-free supernatant was added to a 96-well microtiter plate. Thereafter, 100 µL of 50 mM Tris-HCl (pH 8.0) was added to it. Pyoverdine detection was done by UV spectrophotometry with a range of 350 nm to 600 nm. Spectrometric analysis at 380 nm of pyoverdine production (**A**). Quantification of pyoverdine production (**B**). Error bars indicate the standard error over mean of triplicate experiments. Error bars represent ± SD between three biological replicates. ****corresponds to $p < 0.0001$,

**corresponds to $p < 0.01$, and ns corresponds to $p > 0.05$ based on One-way ANOVA test. Please click here to view a larger version of this figure.

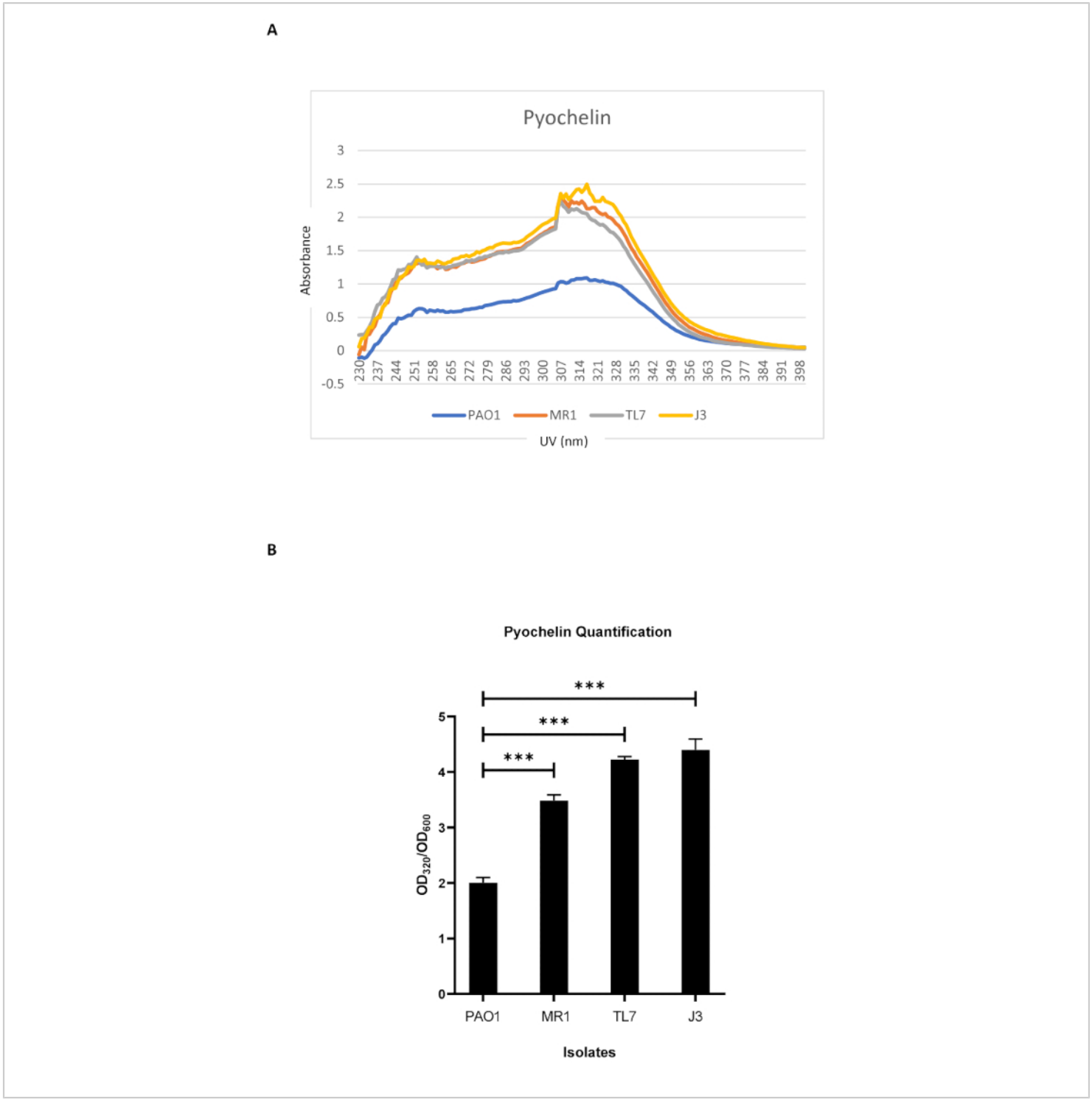

Figure 4: Pyochelin production. Pyochelin production of PAO1 (used as a reference) and clinical isolates (MR1, TL7, and J3) using extraction with ethyl acetate and magnesium sulfate. Spectrophotometric analysis with a range from 230 nm to 400 nm (**A**). Quantification of pyochelin (**B**). Three independent experiments were performed for each isolate. Error bars

represent ± SD between three biological replicates. ***corresponds to $p < 0.001$ based on One-way ANOVA test. Please click here to view a larger version of this figure.

Supplementary Figure 1: Comparative siderophores production in various bacterial growth media. Total siderophores production in four different growth media where King's B media, Peptone water media, and Luria broth were extracted with 3% 8-hydroxyquinoline to remove any trace iron from it, and Luria broth (unextracted) were selected. Extraction by 3% 8-hydroxyquinoline was performed by adding an equal volume of 3% 8-hydroxyquinoline to the media. Error bars represent ± SD between three biological replicates. **corresponds to $p < 0.001$, and ns corresponds to $p > 0.5$ based on One-way ANOVA test.Please click here to view a larger version of this figure.

Supplementary File 1: Media recipes used for the present study.Please click here to view the file.

Discussion

This protocol enables researchers to quantitate total siderophores and two different siderophores of *P. aeruginosa*, namely pyoverdine and pyochelin, from the bacterial cell-free supernatant. In the CAS agar plates assay, CAS dye and Fe^{3+} ions form a complex. When bacteria produce siderophores, they quench Fe^{3+} ions from the CAS-Fe^{3+} complex, leading to a color change around the bacterial growth. This change results in a clear orange halo around the bacterial growth[14,15]. While bacteria can be directly streaked onto CAS agar plates, the overall results may be affected. Streaking a high number of bacterial cells might create a wide orange halo around the bacterial growth, while using fewer bacterial cells might result in a narrow clear zone. To address this, we streaked a uniform number of bacterial cells by adjusting the OD_{600}. Fastidious bacteria may show results in 24 h, while slow-growing bacteria might take more than 72 hours to demonstrate siderophore production.

For the quantitative determination of total siderophores, classical spectroscopy was initially employed, which necessitates a significant volume of supernatant for siderophore detection, making it impractical. The quantification of total siderophores is now conducted using a 96-well plate with a modified CAS assay. This approach has resulted in a reduction in the usage of reagents, bacterial supernatant, time, and increased accuracy compared to traditional spectroscopic methods[16]. Other studies have also reported the use of microtiter plates as a time-saving and more efficient method[17]. The quantification of total siderophores using a microtiter plate has proven to be an efficient approach. In this study, 48-h-old bacterial cultures grown in iron-free media were selected for examination. A comparative study was also conducted to assess siderophore production in different growth media, such as Luria Broth, Luria Broth (extracted with 3% 8-hydroxyquinoline), King's B media (extracted with 3% 8-hydroxyquinoline), and Peptone water media (extracted with 3% 8-hydroxyquinoline). It was observed that Peptone water media exhibited the highest amount of siderophore production, and thus, it was selected for further experiments. A graph depicting siderophore production in different growth media is included in **Supplementary Figure 1.**

Pyoverdine can be detected and quantified using spectrofluorimetric readings, but only pyoverdine is known to exhibit fluorescence. Pyoverdine in complex with Fe^{3+} ions does not exhibit fluorescence[18,19]. For pyoverdine detection and quantification, 48-h-old bacterial cultures were selected

for study. *P. aeruginosa* is known to produce pyochelin under initial iron-starving conditions and switches to pyoverdine production after exposure to severe iron-stress conditions. It has been demonstrated that pyoverdine can chelate other metallic cations, such as aluminum, gallium, manganese, and chromium, but only iron is successfully transported to the cell membrane[19].

Similar to total siderophores and pyoverdine, pyochelin cannot be directly quantified from cell-free supernatant and must be extracted using dichloromethane[20]. Dichloromethane is immiscible with the cell-free supernatant and forms a separate layer at the bottom. In this study, various nutrient media were tested, including Luria broth, Peptone water, and Casamino acid media, but a significant amount of pyochelin was obtained from King's B media. While Hoegy et al. had used CAA media in their protocol, we modified it with King's B media, as CAA media did not support bacterial growth[21]. The method was further modified by extracting nutrient media with 8-hydroxyquinoline to remove any iron residues from the media, creating an iron-deficient medium. Additionally, incubation time was found to significantly affect pyochelin production. Dumas et al. demonstrated that *P. aeruginosa* switches from pyochelin to pyoverdine production under severe iron-limiting conditions[12]. In this study, pyochelin extraction and detection were performed from 24-h-old bacterial cultures, as pyochelin was not detected in 48-h cultures. The volume of the supernatant also affects pyochelin detection, and thus, 100 mL of cell-free supernatant was used for extraction. Furthermore, pyochelin production was significantly higher in MR1, TL7, and J3 isolates, possibly due to bacterial strains that are unable to produce pyoverdine despite having potent iron chelators[22].

Ji et al. introduced a novel and sensitive approach for pyochelin quantification, validating it using LC/MS/MS. However, this method only applied to *P. aeruginosa* isolates from the sputum of cystic fibrosis patients. LC/MS/MS, being a costly setup, may not be affordable for most laboratories[23]. Visaggio et al. developed a bioluminescent whole cell-based biosensor, which enables rapid, sensitive, and single-step pyochelin quantification. Nevertheless, this method cannot provide quantification for total siderophores and pyoverdine[24].

Siderophore iron uptake systems could have an antimicrobial application in drug delivery for multidrug-resistant bacteria, as they play a crucial role in bacterial survival and virulence. This approach may offer a new way to combat multidrug-resistant bacteria[25]. Drugs that cannot penetrate the bacterial cell membrane can be linked to a siderophore, and the resulting Fe-siderophore complex can be transported inside the bacterial membrane[26].

Disclosures

The authors have nothing to disclose.

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