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Antibacterial activity of a mixed-type siderophore belonging to the pyoverdine family produced from *Pseudomonas* sp.

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Abstract: Significant knowledge gaps remain regarding the properties of siderophores and their diverse applications. This study aims to characterize siderophore production by the marine bacterium *Pseudomonas* sp. strain ASA235 and evaluate its effectiveness as an antibacterial agent targeting both Gram-negative and Gram-positive bacteria. Tetrazolium and Arnow's tests revealed that the bacterium produces a mixed-type siderophore belonging to the pyoverdine family. The antimicrobial susceptibility test revealed its activity against *Bacillus subtilis*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*.

keywords: Antibacterial, *Pseudomonas*, Siderophore, Pyoverdine.

1. Introduction

Iron is a vital element required by all living organisms, including animals, plants, and microorganisms such as bacteria and fungi. Its versatility and indispensable contribution to cellular and metabolic processes arise from its involvement in numerous biological activities, including DNA metabolism, methanogenesis, the trichloroacetic acid (TCA) cycle, oxygen transport, and protein activity[1, 2]. It also plays a critical role in the biosynthesis of siderophores, vitamins, toxins, pigments, antibiotics, and aromatic compounds. Moreover, it serves as an essential cofactor for various enzymes and proteins, including nitrogenase, superoxide dismutase, peroxidase, hydrogenase, ribonucleotide diphosphate reductase, and cytochromes[3, 4].

Iron occurs in two oxidation states: ferrous (Fe^{2+}) and ferric (Fe^{3+}). At neutral pH, ferric iron is generally insoluble, forming aggregates of ferric hydroxide polymers that limit its bioavailability to organisms despite its abundance[5]. The chelation strategy is among the approaches bacteria rely on to grow under iron starvation conditions[6].

Siderophores, iron chelators, are small, low-molecular-weight secondary metabolites produced by microorganisms under iron-deficient conditions[7, 8]. These molecules are secreted to scavenge iron by chelating it from both soluble and insoluble iron complexes, including ferric transferrin, ferric citrate, and ferric phosphate. Moreover, siderophores can also chelate iron from synthetic chelators like EDTA[9].

Fluorescent pseudomonads of the genus *Pseudomonas*, a group of Gram-negative bacteria known for their ubiquity and metabolic versatility, are among the prominent siderophore-producing microorganisms. Extensive research has focused on these bacteria's iron stress response mechanisms, particularly under iron-limiting conditions. Notably, fluorescent pseudomonads are well-known for producing siderophores belonging to the pyoverdine (PVD) family, which are among the most complex and extensively studied[10].

In this study, an attempt was made to explore the ability of *Pseudomonas* sp. to produce a siderophore that has been characterized, purified, and used as an antibacterial agent.

2-Materials and methods

All chemicals used in preparing the Chrome azurol S (CAS) reagent were provided by Sigma-Aldrich. All buffers and reagents are prepared using distilled de-ionized (DI) water. Glasswares were rinsed with 6 N HCl to remove iron traces before use.

1-The Microorganism and growth conditions

The bacterial strain of this study was previously isolated from a deep Red Sea soil sediment sample collected in the Red Sea near Hurghada, Egypt. Molecular identification confirmed the strain as *Pseudomonas* sp. strain ASA235, and it was subsequently submitted to the GenBank database, where it received the accession number MH580294. The test strain was routinely cultured in Luria-Bertani (LB) broth media containing (g/l): 10g peptone, 5g yeast extract, and 10g NaCl and incubated at 37°C for 24 hours, and then preserved at -20°C in 30% glycerol.

2-Quantitative assays for siderophore production

The CAS liquid indicator solution was prepared using four components: solution 1 (0.009 g CAS in 40 mL DI H_2O), solution 2 (0.016 g $FeCl_3$ in 100 mL DI H_2O), solution 3 (0.017 g HDTMA in 40 mL DI H_2O), and solution 4 (5.95 g HEPES buffer in 10 mL DI H_2O, pH 5.6). Solution 1 was mixed with 1.5 mL of solution 2, followed by adding solution 3 with gentle mixing. Finally, solution 4 was added, and the total volume was adjusted to 100 mL with DI H_2O[11, 12].

The strain was cultured in iron-free media (MM9) at 37°C, 150 rpm for 24 h. After centrifugation at 6000 rpm for 20min, 1 mL of the supernatant obtained was mixed with 1 mL of the CAS reagent. Color changes in the CAS solution were measured spectrophotometrically at 630 nm, and the results were expressed as siderophore units (SU%) according to the following formula:

$$\% \text{ Siderophore Unit} = [(Ar - As) / Ar] \times 100 \quad \text{(Eq. 1)}$$

Where Ar represents the absorbance of the reference measured at 630 nm (CAS reagent with uninoculated MM9 media), while As represents the absorbance of the sample at the same wavelength [13].

3-Detection of function type of siderophore

The nature of the siderophore was characterized using chemical tests. Tetrazolium and Arnow's tests were used to discriminate between hydroxamate and catecholate type siderophores [14, 15], which is confirmed by $FeCl_3$ [16], while Shenker's test was used to determine carboxylate siderophores [17]. *Pseudomonas* sp. was incubated at 37°C, 150 rpm for 24 h in MM9 broth medium, and then the previous experiments were conducted using the cultural supernatant.

4-Purification of siderophore

Pseudomonas sp. was inoculated on iron-free MM9 broth medium containing (g/l) 3 g asparagine, 4 g glucose, 0.4 g NaCl, 0.3 g KH_2PO_4, 0.493 g $MgCl_2.6H_2O$, 0.055 g $CaCl_2$, and 3 g yeast extract. The culture was incubated at 28°C, 150 rpm for 72 h. After centrifugation at 6000 rpm for 30 min, the siderophore was purified following the method described by Srivastava, Sahgal [18].

5-Antibacterial activity of the purified pyoverdine

The antibacterial activity of pyoverdine extracted from *Pseudomonas* sp. was measured on Muller-Hinton agar medium using the agar well diffusion technique against four clinical isolates (obtained from Cairo MERCIN, Faculty of Agriculture, Ain Shams University, Cairo, Egypt): two Gram-negative bacteria, *Escherichia coli* (ATCC 10536) and *Klebsiella pneumoniae* (ATCC 10031) and two Gram-positive bacteria, *Bacillus subtilis* (DMS 1088) and *Staphylococcus aureus* (ATCC 6538). The surface of the agar plate was inoculated by evenly spreading a specific volume of the bacterial inoculum across the entire agar surface. A well, 8 mm in diameter, was aseptically punched, and 100 µL of the sample with a concentration of 100 mg/mL was added to the well. After an incubation period of 18 to 24 hours at 37°C, the plates were examined for inhibition zones, which were measured and recorded[19].

Results

1-Quantitative assays for siderophore production

Changing the color of the liquid CAS indicator from blue to yellow, as shown in Figure (1), indicates positive results for siderophore production. This strain demonstrated the ability to produce 44.5% siderophore units, which was calculated acchording to Eq1.

$$\% \text{ Siderophore Unit} = [(0.961 - 0.533) / 0.961] \times 100.$$

Figure (1): The CAS liquid assay of *Pseudomonas* sp. **(A)** Control showed no color change, indicating a negative result. **(B)** Formation of the yellow color of the bacterial siderophore indicating positive CAS test.

2-Detection of siderophore function type

Tests conducted to detect the chemical nature of the siderophore, as shown in Figure (2), yielded positive results for both the tetrazolium test, which exhibited a characteristic red color, and Arnow's test, which displayed a distinct deep yellow-brown color. Additionally, the $FeCl_3$ test revealed a characteristic maximum absorbance at 468 nm. These results, along with a characteristic maximum absorbance at 468 nm in the $FeCl_3$ test, suggest that the type of siderophore is likely of a mixed type "containing both hydroxamate and catecholate group.

3-Antibacterial activity of the purified pyoverdine

The purified pyoverdin from *Pseudomonas* sp. was tested for antibacterial activity against a range of Gram-positive and Gram-negative bacteria, as shown in Table (1) and Figure (3). Pyoverdin exhibited antibacterial activity against the Gram-negative bacterium *Klebsiella pneumoniae*, as well as the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, but it showed no effect against *Escherichia coli*. This study used Azithromycin antibiotic (2 mg/ml) as the standard control.

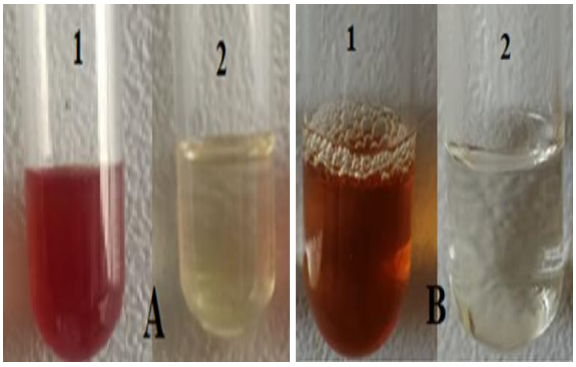

Figure (2): Chemical characterization of the siderophore produced by *Pseudomonas sp.* **(A1)** The formation of a deep red color indicated a positive result in the tetrazolium test, whereas **(A2)** the control showed no color change, confirming a negative result. Similarly, **(B1)** the appearance of a deep yellow-brown color confirmed a positive result in Arnow's test, while **(B2)** the control exhibited no color change, indicating a negative result.

Table (1): Antibacterial activity of the purified pyoverdine.

Examined bacterial strains	Inhibition zones in mm	
	Purified pyoverdin (100 mg/ml)	Azithromycin antibiotic (2 mg/ml)
Klebsiella pneumoniae (ATCC 10031)	21.5 ± (0.353)	22 ± (0.707)
Escherichia coli (ATCC 10536)	-	17 ± (0.707)
Staphylococcus aureus (ATCC 6538)	24.5 ± (1.060)	27 ± (1.414)
Bacillus subtilis (DMS 1088)	25 ± (0.707)	24 ± (0.707)

The values listed are the mean values of duplicate replicates ± SE.

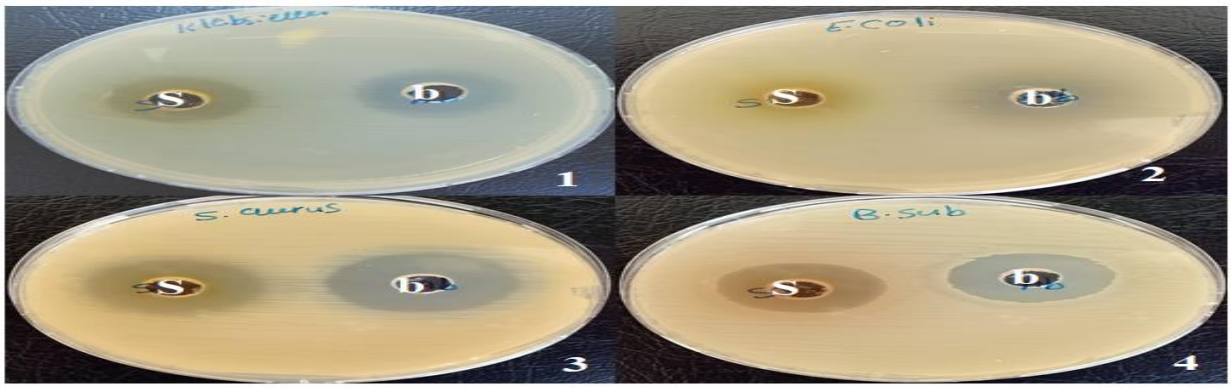

Figure (3): Antibacterial effect of (**S)** the purified pyoverdin, and **(b)** standard Azithromycin antibiotic against **(1)** *Klebsiella pneumoniae*, **(2)** *Escherichia coli*, **(3)** *Staphylococcus aureus*, and **(4)** *Bacillus subtilis*.

Discussion

The worldwide rise in antibiotic resistance underscores the critical need for novel drugs to target bacterial pathogens. A widely adopted approach involves exploring natural compounds produced by microbes to suppress their competitors.

This study reveals that pyoverdines, iron-chelating siderophores synthesized by environmental *Pseudomonas* species, possess significant antibacterial activity by inducing iron deprivation and inhibiting the growth of pathogens.

Pseudomonas sp. is among the most well-known Gram-negative bacteria for siderophore production, with pyoverdine being its most prominent siderophore[20, 21]. The ability of *Pseudomonas* sp. ASA235 to produce a considerable amount of siderophores (44.5%), a result consistent with findings reported by Sasirekha and Srividya [22] and Colombowala and Aruna [23].

Although there is a diversity of siderophore-producing bacteria, the chemical groups that bind Fe^{3+} are conserved. Most siderophores possess hydroxamate and/ or catecholate groups involved in binding with iron. The tested *Pseudomonas* sp. was found to produce a mixed-type siderophore, as confirmed by positive results in both tetrazolium and Arnow's tests. The results obtained in Arnow's test may be attributed to the substitution of vicinal diols in positions 3 and 4, leading to the formation of an unstable yellow compound as a final product of this reaction[24]. The λmax obtained at 468 nm, neither 420-450 for hydroxamate nor 495 nm for catecholate, indicated that the type of siderophore might be a mixed-type "containing hydroxamate and catecholate group". The same results have been obtained from other species of *Pseudomonas* [24].

Our results indicated the activity of pyoverdine against human pathogens *Staphylococcus aureus*, *Bacillus subtilis*, and *Klebsiella pneumoniae*. These findings were similar to the result of [19]. Liu, Dai [25] reported that pyoverdine (PVD) can be used as an antibacterial agent due to its inhibitory effect against both Gram-negative and Gram-positive bacteria. Furthermore, it was stated that, as a biocontrol agent, PVD not only competes for iron but also directly inhibits the growth of pathogenic bacteria.

Conclusions

The findings in this study demonstrate that under iron-limited conditions, *Pseudomonas* sp. strain ASA235 could produce a mixed-type siderophore from the pyoverdine family, which has a promising capability to have an inhibitory effect with a broad spectrum against Gram-negative and Gram-positive bacteria. Consequently, pyoverdine has the potential to be used as an effective agent for bacterial infection management.

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