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Black-Pigmented Marine *Pseudomonas aeruginosa* Exhibiting Anti-Bacterial Activity against Multidrug-Resistant (MDR) Wound Infection Bacteria

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ABSTRACT

The urgency of multidrug-resistant (MDR) bacterial infections in wounds is a significant concern due to the high prevalence of MDR in healthcare settings. Black pigmented marine bacteria, strain PS1C, were isolated from marine sediment on Awur Beach Jepara, Central Java, Indonesia, and cultured in the laboratory. The aim of this research includes molecular identification of strain PS1C, extracting black pigment from strain PS1C, isolation of MDR bacteria from wounds, and evaluating the antibacterial activity of black pigment from strain PS1C against MDR bacteria isolates of wound infections. We examined the 16S rRNA gene sequences strain PS1C to identify the species. Then, the black pigment from PS1C was extracted using methanol: acetone (7:3) solvent. Antibacterial activity was evaluated against MDR bacteria from wounds with the microdilution method. A black-pigmented bacterium was isolated and identified as *Pseudomonas aeruginosa* PS1C. We found that the black pigment from *P. aeruginosa* PS1C can be developed as an antibacterial agent against the MDR bacterial isolate of wounds with MIC and MBC values of 6.25-12.5 mg/ml and 6.25-25 mg/ml, respectively. In conclusion, the study's findings highlight the potential of the extract of black pigment from *P. aeruginosa* PS1C as an antibacterial agent against wound-causing MDR bacteria and reinforce previous research into *P. aeruginosa* can be isolated from marine sources. Additional *in vivo* investigations and the identification of the antibacterial activity's mode of action are required.

1. Introduction

Wound infections occur when bacteria or other pathogens infiltrate a wound, producing inflammation and perhaps delaying or preventing healing. The infection's severity can range from localized, where the bacteria cause damage to the wound tissues, to systemic, where the infection spreads throughout the body and causes major health concerns (Liu *et al.* 2022). The incidence of wound infection varies according to the patient group and kind of wound. In a study of patients with wounds, the rate of wound infection was 5.95% (Woo *et al.* 2022). An estimated 157,000 Surgical site infections (SSIs) occurred in US acute care hospitals in 2011, and 3% of patients with SSIs died as a result of the infection (Umscheid *et al.* 2011).

The prevalence of wound infections in Indonesia is not specifically stated in the search results. However, one study discovered a reported Healthcare-associated infection (HAI) frequency of 7.1% at two academic medical centers in East Java (Budayanti *et al.* 2020). Another investigation looked at the norms and patterns of antibiotic prescribing in Indonesian hospitals using a multicenter point prevalence analysis. It was discovered that 26.8% of hospital-acquired infections were treated with targeted antibiotic therapy (Limato *et al.* 2021). These studies suggest that the prevalence of wound infections in Indonesia is significant.

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A severe threat to public health is posed by multidrug resistance (MDR) bacterial wound infections. It can lead to longer hospital admissions, higher treatment costs, and the spread of resistant strains of the bacterium. Common bacteria in wound infection, such as *S. aureus, P. aeruginosa, E. coli, Acinetobacter* spp., and *Klebsiella* spp. are frequently the cause of these infections. These bacteria can resist various classes of antibiotics, not only aminoglycosides, quinolones, and third and fourth-generation cephalosporins (Pîrvănescu *et al.* 2014) but also vancomycin (Cong *et al.* 2020). Globally and regionally, there are differences in the incidence of MDR bacteria in wound infections; developing countries like Ethiopia, Nigeria, and Ghana have been found to have high rates of MDR infections (Morshad *et al.* 2021). The excessive and improper utilization of antibiotics can lead to genetic modifications in bacteria, resulting in a reduction of the efficacy of various antibiotics and expediting the urgency of multidrug-resistant (MDR) bacteria (Nobel *et al.* 2022). Therefore, the development of effective treatment strategies is important, one of which is developing natural antibacterial agents from biological sources.

The natural anti-MDR bacterial agents can be derived from latex (Prastiyanto *et al.* 2020b), seeds (Prastiyanto 2021), fruits (Prastiyanto *et al.* 2020c, 2021), mushrooms (Prastiyanto *et al.* 2020a), lactic acid bacteria (Lestari *et al.* 2019) and marine bacteria (Prastiyanto *et al.* 2022b, 2023). In recent years, research into marine bioactive chemicals has revealed numerous therapeutic possibilities. The bioactive potential of marine sources efficiently combats human infections (Blunt *et al.* 2017). Research on the antibacterial activity of marine bacterial isolates has provided interesting assumptions that most pigmented bacteria have antibacterial activity against human pathogens (Offret *et al.* 2016). One of the pigments is melanin. Melanin is a pigment produced by organisms throughout all domains of life, including marine bacteria. This pigment can be black or brown.

Marine Pseudomonas species, such as *P. stutzeri*, can produce melanin pigments. According to a study by Kumar *et al.* (2013), the marine *P. stutzeri* strain HMGM-7 [MTCC 11712] produced 6.7 g/L of melanin at a significant rate. *P. otitidis* DDB2 has the potential to produce melanin, although no antibacterial action was identified against tested pathogens (Deepthi *et al.* 2021). Another Investigation has documented the potent antibacterial properties of melanin synthesized by *P. balearica against S. aureus, E. coli*, and *Candida* (Zerrad *et al.* 2014). Lately, we isolated one marine bacterial strain that has black pigment characteristics. The strains were designated *P. aeruginosa* PS1C according to a 16S rRNA sequence phylogenetic analysis. Here, we state bacterial isolation, black pigment extraction, and antibacterial efficacy against MDR bacteria isolate wound infection.

2. Materials and Methods

2.1. Sample Collection

Marine sediment was collected approximately from a depth of 1.5 m from Awur Beach Jepara, Central Java, Indonesia, on April 11, 2023, at 6°36'17.8"S 110°38'34.8"E (Figure 1). Following collection, the samples were submerged in sterile bags, refrigerated at 4°C, and transported to the lab (Prastiyanto *et al.* 2022b).

2.2. Marine Bacterial Isolation and Molecular Identification

2.2.1. Bacterial Isolation and Purification

Nine milliliters of sterile seawater were combined with one gram of crushed marine debris. The sediment sample was diluted using a 10^{-4} dilution solution. Subsequently, it was promptly distributed into Petri dishes with Zobell agar medium, which consisted of 0.5 g yeast, 2.5 g peptone, and 13 g Bacto agar in 1 L of seawater. The mixture was then incubated for three days at a temperature of 28±2°C (Prastiyanto *et al.* 2022b, 2022c). On Petri dishes, bacterial colonies grew following a 3-day incubation period. After being separated using the streak plate method, the black-pigmented bacteria were grown for three days at 28±2°C in a fresh medium. Based on factors like size, color, margin, elevation, and shape, the pigmented bacteria were purified. Up till the colony was free of contaminants, the purification procedure was repeated. For additional examination, the refined bacteria were kept in storage at 10°C.

2.2.2. Culture of Bacteria

The pure bacteria were cultivated using Zobell broth medium. The bacteria culture was conducted at a temperature of 28±2°C in a shaking incubator for twenty-four hours. The cellular precipitation of

Figure 1. Map of the study areas in Awur Beach, Jepara, Central Java, Indonesia. A-E, Sampling sites

the bacteria was carried out using centrifugation at 6,000 rpm in a 4°C centrifuge for 15 minutes.

2.2.3. Molecular Identification

The extraction of DNA from bacterial cells was done using PrestoTM Mini DNA Bacteria Kit following the guidelines provided by the manufacturer. The ultimate elution volume employed is 50 µL. The DNA was kept at a temperature of 4°C until it was needed for PCR. The bacterial DNA concentration used in this study is 50 ng/µL, and a mixture of 2 µL of bacterial DNA is combined with a 16S rRNA gene primer. Primer 27F '5'-AGAGTTGATCMTGGCTCAG-3' and 1492R '5'-CGGTTACCTTGTTACGACTT-3' were utilized, with a final primer concentration of 10 µM. According to the PCR amplification technique, the Taq polymerase enzyme must be activated for four minutes at 95°C. Thirty-five cycles of denaturation (at 95°C for 30 seconds), primer annealing (at 57°C for 30 seconds), extension (at 72°C for 2 minutes), further extension (10 minutes) at 72°C, and cooling down (10 minutes) at 4°C come next. The PCR products are then separated on a 0.8% agarose gel, and Flourovue is used to identify unique DNA bands. The analysis of 16S rRNA gene sequences by Genetika Science Tangerang is conducted using PCR product sequencing. The Basic Local Alignment Search Tool (BLAST) database tool at the National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) (Altschul *et al.* 1990).

2.2.4. Phylogenetic Analysis

The technique makes use of the phylogenetic analysis program MEGA X. The Phylogenetic tree was reconstructed using MEGA X software. ClustalW was used to align the 16S rRNA gene data sequence. The generation of phylogenetic trees was conducted with the neighbour-joining, employing the Tamura-Nei model. A non-parametric bootstrapping technique was conducted on 1,000 datasets derived from 16S rRNA gene sequences to construct the trees. The connections between closely related strains are demonstrated by these evolutionary relationships (Kumar *et al.* 2018).

2.3. Extraction of Pigment

The pigment extraction procedure is similar to the melanin purification procedures described in earlier research (Zerrad *et al.* 2014), with some adjustments. The obtained cells were homogenized using a

mixture of methanol and acetone (7:3, v/v), with 1 ml of mixture per 0.1 g of cells, then the mixture was vortexed 5 times, Each vortexing consisted of 1 minute of vortexing followed by 1 minute on ice (Setiyono *et al.* 2020). After that, the cells were subjected to ten minutes of sonication in pulse mode with 60% amplitude and 10 s on/30 s off (QSonica, Newtown, Connecticut, USA). A small amount of $CaCO_3$ and sodium ascorbate were included in the mixture to stop the oxidation-induced deterioration of the pigment. To separate the pigment extract from the cell debris, centrifugation was performed at 10,000 g for 15 minutes at 4°C. After that, the extract was gathered and dried with nitrogen gas. Dried pigment extract was kept at -30°C.

2.4. Isolation, Identification, and Screening for Antibacterial Activities against MDR Bacteria Isolate from the Wound

Wound samples obtained from patients at Gunung Jati Hospital in Cirebon, Indonesia, were utilized to acquire bacterial isolates. The bacterial specimens were cultivated on Blood and MacKonkey agar medium and subjected to a 24-hour incubation period at a temperature of 35±2°C. The susceptibility to microorganisms was assessed using Vitek®MS (Prastiyanto *et al.* 2021).

2.5. Antibacterial Activities against MDR Bacteria

2.5.1. Minimum Inhibitory Concentration (MIC)

The microdilution method determines the MIC by adding 0.05% 2,3,5-Triphenyl tetrazolium chloride to Mueller Hilton Broth (MHB) medium in a microwell plate (Prastiyanto 2021). Initially, 100 µL of MHB was put into each well, and then 100 µL of pigment was added to the first well. Dilution in series went on to the twelfth well. Following that, 10 µL of MDR bacteria were added to each well with 0,5 McFarland (1.5×10^8 CFU/ml) density and they were incubated at 35±2°C for 18–20 hours.

2.5.2. Minimum Bactericidal Concentration (MBC)

MIC wells were subcultured on Blood Agar Plate (BAP). By tracking bacterial growth on the BAP medium after incubation at 35±2°C for 16–20 hours, the MBC value was determined. The lowest concentration that prevents MDR bacteria from thriving is used to calculate the MBC value (Prastiyanto *et al.* 2022a).

3. Results

3.1. Bacterial Isolation

A single strain was successfully isolated from marine sediment. Here are the characteristics of the growing bacteria on Zobell marine agar medium (incubated for 72 hours at 28±2°C) that produce black pigment (Figure 2A), a non-fermentative gram-negative rod with positive catalase and oxidase characters. PS1C bacteria on Zobell marine broth medium were incubated for 72 hours at 28±2°C (Figure 2B). PS1C bacterial pellet from a 6,000 rpm centrifuge at 4°C for 15 minutes (Figure 2C).

3.2. Molecular Identification of PS1C Strain

The 16S rRNA gene sequences of strains PS1C contained 1,410 bp of nucleotides. This isolate has been registered in GenBank with an accession number PP754996. The nucleotide BLAST program (NCBI) for highly similar sequences was used to evaluate the similarity between these bacteria's 16S rRNA genes. A comparative analysis of the 16S rRNA gene sequences revealed that *P. aeruginosa* strain ATCC 10145 and strain PS1C shared 100% of the percent identity. *P. aeruginosa* strain ATCC 10145 and strain PS1C were grouped in the neighbor-joining tree phylogeny, which had a 98% bootstrap resampling value (Figure 3B).

3.3. Growth Kinetics and Pigment Yield

The results of bacterial culture using Zobell broth medium are then visualized as a curve. The growth kinetics curve of *P. aeruginosa* PS1C, which compared cell mass obtained against time, showed that the log phase began after 4 hours and ended at roughly 48 hours when the stationary phase began and 52 hours when the biomass began to decrease. The maximum pigment, which weighed 3.05 g, was collected 48 hours after the production of the pigment began at 24 hours. The production kinetic study revealed that the maximum concentration of cell biomass, 5.8625 g, was achieved following a 48-hour incubation period (Figure 4).

3.4. MDR-bacteria from Wound

The results showed that the bacteria isolated from wounds were *S. aureus, E. coli, P. aeruginosa, K. pneumoniae*, and *E. faecalis*. MDR bacteria from wound samples included Methicillin-Resistant *S. aureus* (MRSA), MRSA + vancomycin-

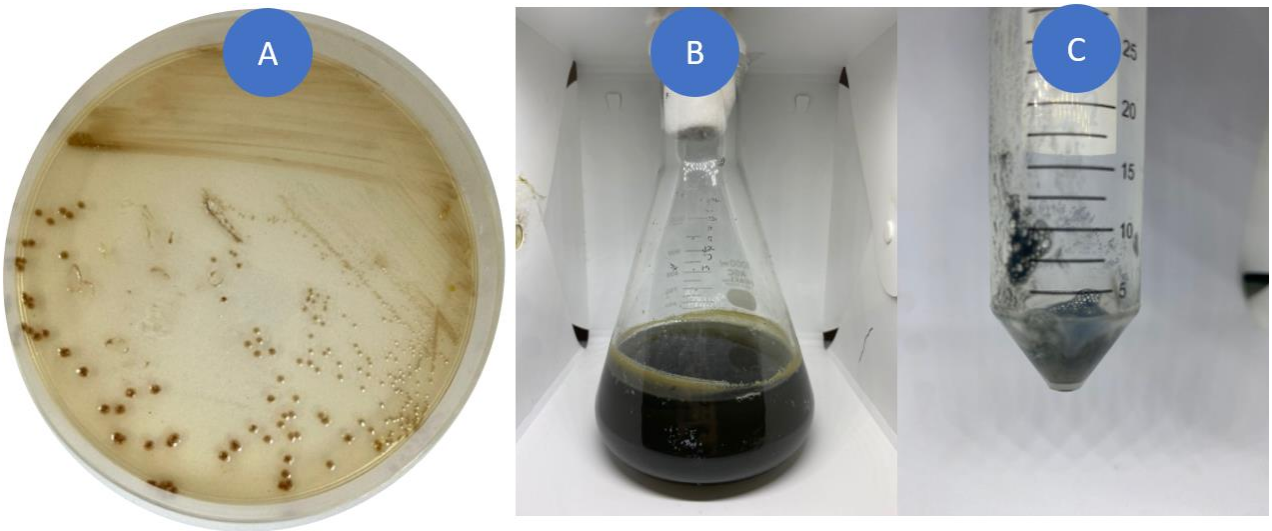
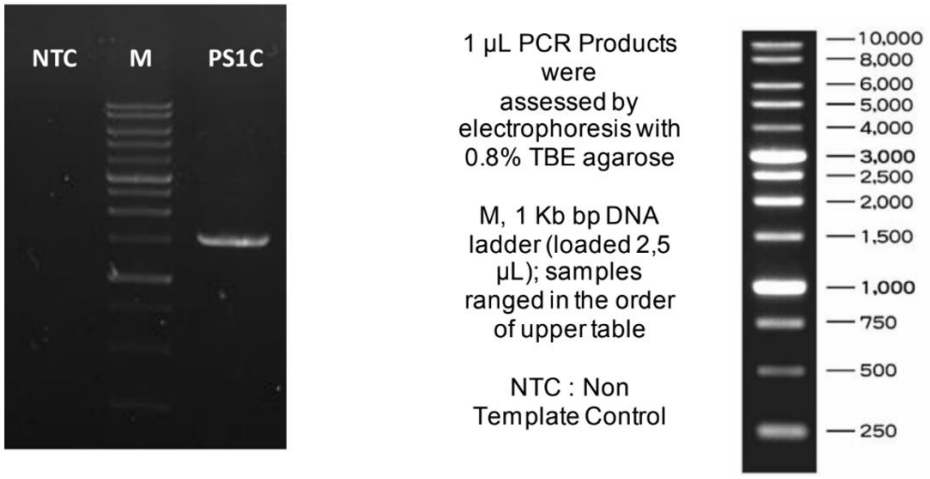


Figure 2. Isolated strains, PS1C. (A) PS1C bacterial colonies, (B) PS1C bacteria on zobell marine broth, (C) PS1C bacterial pellet

A



B

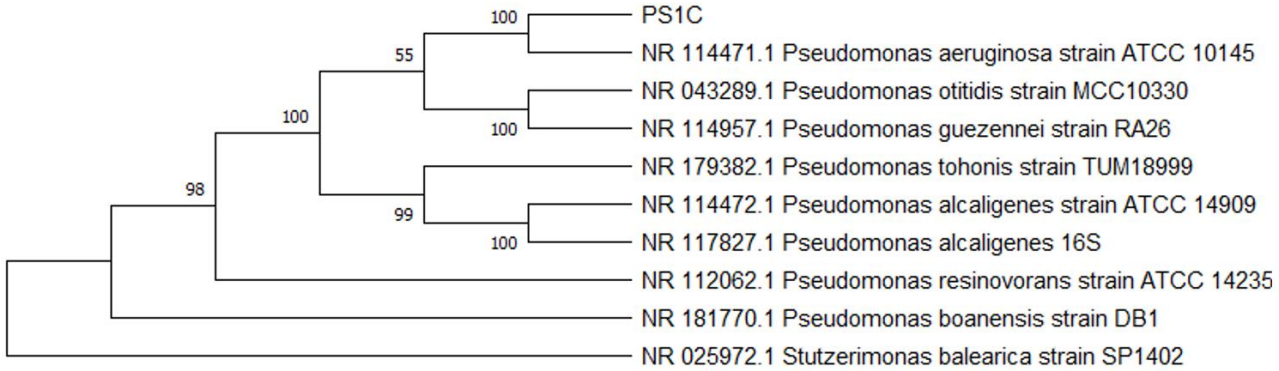


Figure 3. (A) 16S rRNA gene PCR amplicons on 0.8% agarose gel of Pseudomonas aeruginosa strains PS1C, (B) phylogeny of strains PS1C, the type strains of recognized species in the genus *Pseudomonas*, and representatives of related taxa. Stutzerimonas balearica was used as an outgroup. Only bootstrap values >50% (expressed as percentages of 1000 replications) are shown at branch points

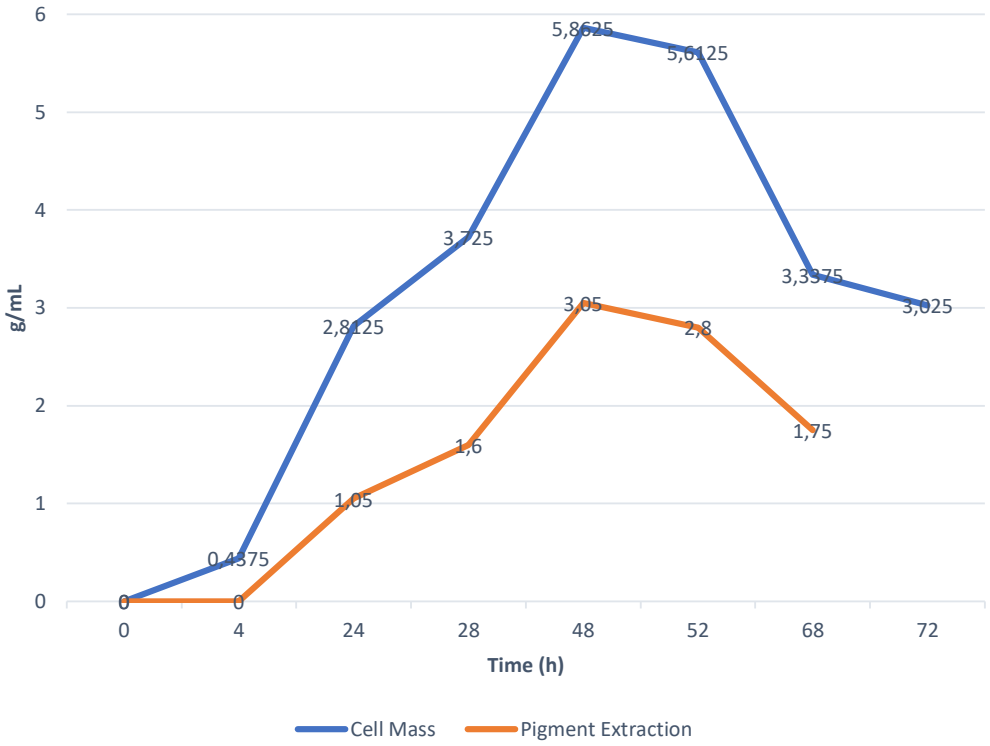


Figure 4. *Pseudomonas aeruginosa* PS1C growth kinetics and production of pigments kinetics

resistant *S. aureus* (VRSA), extended-spectrum β-lactamase-producing *Escherichia coli* (ESβL-E. coli), Carbapenem-resistant *P. aeruginosa* (CRPA), Carbapenem-Resistant *K. pneumoniae* (CRKP), and vancomycin-resistant *E. faecalis* (VRE) (Table 1).

3.5. Antibacterial Activities against MDR Bacteria from Wound Infection

The minimum inhibitory concentration (MIC) of *P. aeruginosa* PS1C pigments against MDR-bacteria from wound samples is obtained using a microwell plate and the microdilution method (Figure 5). The pigments' MIC values, according to the data, varied from 6.25 mg/ml to 12.5 mg/ml. Pigment showed better activity against non-vancomycin-resistant MDR bacteria because it had a lower MIC value of 6.25 mg/ml. In comparison, MDR bacteria with vancomycin-resistant (MRSA+VRSA and VRE) pigment showed antibacterial activity at a concentration of 12.5 mg/ml. *P. aeruginosa* PS1C pigment showed better results against MRSA, ESBL, and Carbapenem-resistant bacteria than against Vancomycin-resistant bacteria because it has a lower MBC value of 6.25-12.5 mg/ml. In comparison, in vancomycin-resistant bacteria the pigment MBC value is 25 mg/ml (Figure 6).

4. Discussion

PS1C strains were successfully isolated from marine sediment. Molecular identification showed that strains PS1C were grouped with *P. aeruginosa.* Research reports related to *P. aeruginosa* in marine habitats are still very limited. Kimata *et al.* (2004) first reported the species indigenous adaptability in marine habitats, which had been a source of contention (Kimata *et al.* 2004). Other studies have also shown that *P. aeruginosa* can be isolated from the open ocean and coastal areas (Khan *et al.* 2007; Nonaka *et al.* 2010). The results of this study reinforce previous research reports that *P. aeruginosa* can be isolated from marine sources.

P. aeruginosa PS1C produce black pigment. However, this study did not detect black pigment compounds. However, previous research reports show that the black pigment from *Pseudomonas* is pyomelanin. *P. aeruginosa* can produce a black pigment known as pyomelanin. The production of pyomelanin by *P. aeruginosa* is an uncommon characteristic in the genus. Pyomelanin is not typically seen in *P. aeruginosa* strains, and its production is less ubiquitous than that of pyocyanin (Hunter and Newman 2010). At the time of writing

Table 1. The results of identification and Susceptibility test of antibiotics MDR bacteria isolated from wound

Species and code strain	Common name of MDR bacteria	Antibiotic resistance pattern
Staphylococcus aureus SA1	MRSA	Benzylpenicillin, Oxacillin, Gentamicin, Ciprofloxacin, Levofloxacin, Moxifloxacin, Nitrofurantoin,
Staphylococcus aureus SA2	MRSA	Benzylpenicillin, Oxacillin, Gentamicin, Ciprofloxacin, Levofloxacin, Moxifloxacin, Nitrofurantoin,
Staphylococcus aureus SA3	MRSA	Benzylpenicillin, Oxacillin, Gentamicin, Ciprofloxacin, Levofloxacin, Moxifloxacin, Nitrofurantoin,
Staphylococcus aureus SA5	MRSA+VRSA	Benzylpenicillin, Oxacillin, Gentamicin, Ciprofloxacin, Levofloxacin, Moxifloxacin, Nitrofurantoin, Vancomycin
Staphylococcus aureus SA6	MRSA+VRSA	Benzylpenicillin, Oxacillin, Gentamicin, Ciprofloxacin, Levofloxacin, Moxifloxacin, Nitrofurantoin, Vancomycin
Staphylococcus aureus SA4	MRSA	Benzylpenicillin, Oxacillin, Gentamicin, Ciprofloxacin, Levofloxacin, Moxifloxacin, Nitrofurantoin,
Escherichia coli EC1	ESβL	Ampicillin, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, Aztreonam, Ciprofloxacin, Nitrofurantoin Sulfamethoxazole
Pseudomonas aeruginosa PA1	CRPA	Ampicillin, Sulbactam, Tazobactam, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, Aztreonam, Meropenem, Amikacin Gentamicin, Ciprofloxacin, Tigecycline, Nitrofurantoin, Sulfamethoxazole
Klebsiella pneumoniae KP1	CRKP	Ampicillin,Sulbactam,Tazobactam,Cefazolin,Ceftazidime, Ceftriaxone, Cefepime, Aztreonam, Ertapenem, Meropenem, Ciprofloxacine, Sulfamethoxazole
Enterococcus faecalis EF1	VRE	Gentamicin, Streptomycin, Ciprofloxacin, Levofloxacin, Erythromycin, Vancomycin, Tetracycline

MRSA: Methicillin-Resistant *Staphylococcus aureus*, VRSA: vancomycin-resistant *Staphylococcus aureus*, ESβL: extended-spectrum β-lactamase, CRPA: Carbapenem-resistant *Pseudomonas aeruginosa*, CRKP: Carbapenem-Resistant *Klebsiella pneumoniae*, VRE: vancomycin-resistant *Enterococcus*

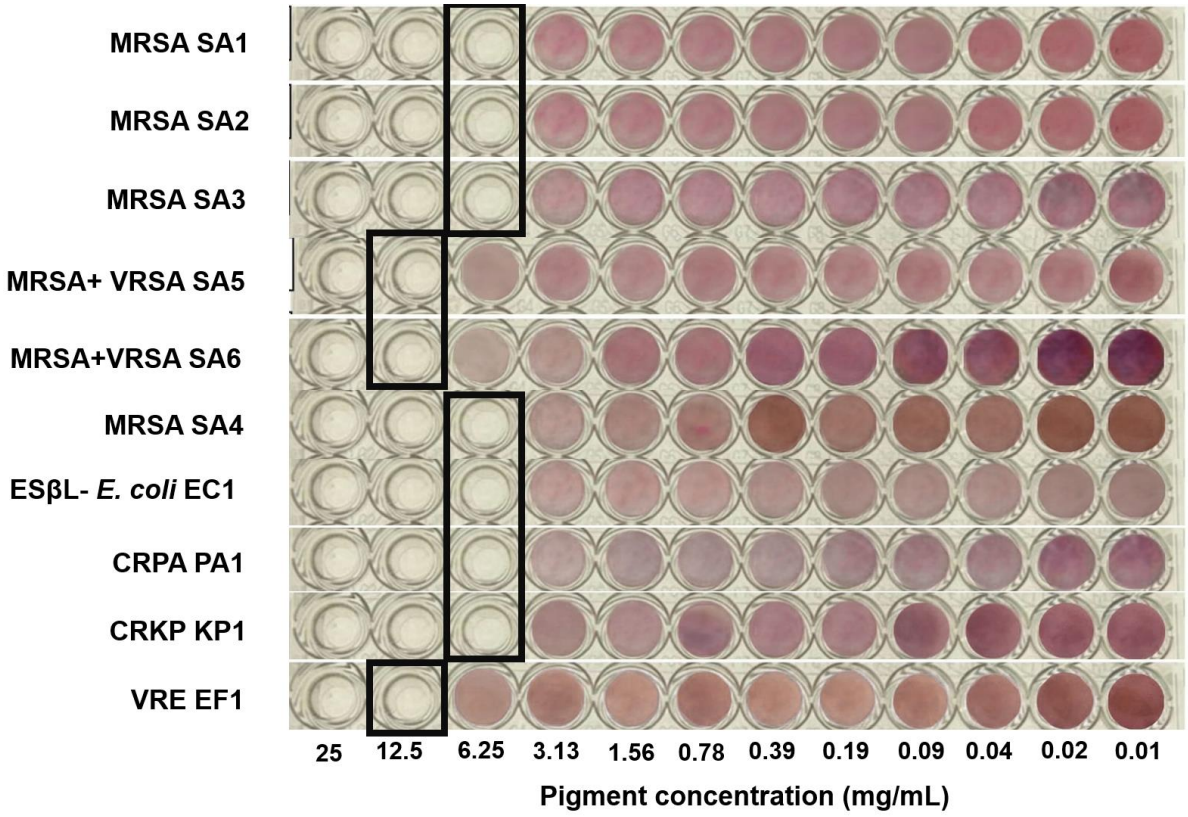


Figure 5. MIC values of pigment against MDR bacteria

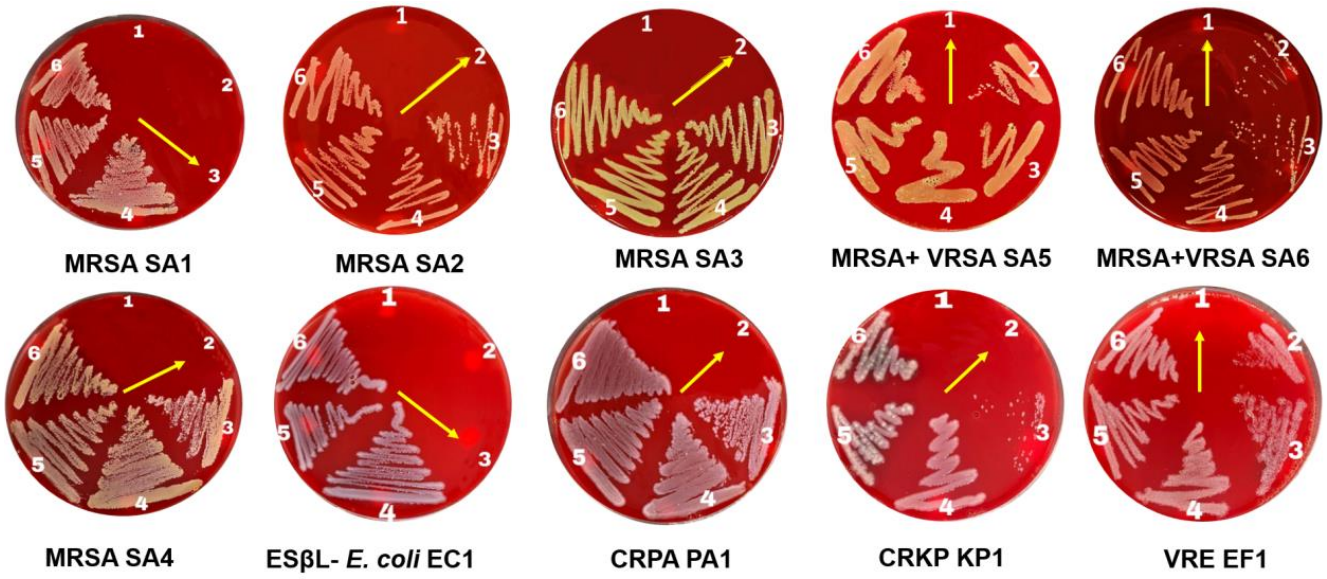


Figure 6. MBC values of pigment against MDR bacteria. Concentrations: 1). 25 mg/ml, 2). 12.5 mg/ml, 3). 6.25 mg/ml, 4). 3.13 mg/ml, 5). 1.56 mg/ml, 6). 0.78 mg/ml, Arrow: MBC values

this report, research reports related to pyomelanin from *P. aeruginosa* were derived from non-marine isolates (Hunter and Newman 2010; Hocquet *et al.* 2016; Bolognese *et al.* 2019). Therefore, the results of this study provide new information that *P. aeruginosa* strain PS1C marine isolates also produce black-colored pigments.

Based on the kinetic analysis, it was determined that the strain grew fast because the cell mass productivity was only 0.122 g/h. The specific pigment production rate was 0.52 grams of pigment produced for every gram of biomass. After 48 hours of incubation under ideal circumstances, 1 L of the strain culture generated 5.8625 g of biomass in total for the yield analysis. The solvent extraction process using methanol: acetone (7:3) recovered a total of 3.05 g of crude pigment upon the vaporization of the solvent from the extraction mixture. This indicated a crude pigment extraction yield from biomass of 52.02%. The pigment yield obtained was both lower and higher than other reported microorganism pigments (Table 2). The pigments obtained were evaluated for their antibacterial potential against MDR bacteria from wound samples.

This result showed that the bacteria that were found in wounds were *S. aureus, E. coli, P. aeruginosa, K. pneumoniae*, and *E. faecalis*. This outcome is in line with earlier studies. Typical MDR bacteria that cause wound infections include *P. aeruginosa, S. aureus, K. pneumoniae, E. faecalis*, and *E. coli* (Puca *et al.* 2021). These bacteria are frequently resistant to multiple antibiotics, making them difficult to treat and increasing the chance of antibiotic failure, which can result in increased mortality (Ahmed *et al.* 2023). The prevalence of MDR bacteria in wound infections is a global issue, with more than 90% of MRSA (Ahmed *et al.* 2023). The isolation results of MDR bacteria from the wound samples obtained were then used as test bacteria to evaluate the antibacterial potential of the pigments.

According to the findings, *P. aeruginosa* PS1C pigments have antibacterial activity against MDR bacteria from wound isolates. *P. aeruginosa* PS1C pigment showed antibacterial activity results against MRSA, ESBL, and Carbapenem and Vancomycin-resistant bacteria. The results of this study are better than those of previous studies. *P. otitidis* DDB2 produces melanin, but no antibacterial activity was detected against tested pathogens (Deepthi *et al.* 2021). On the other hand, reports showed that melanin isolated from *P. balearica* showed strong antimicrobial activity against *S. aureus, E. coli*, and *Candida* (Zerrad *et al.* 2014). However, the study only used the diffusion method so that the smallest concentration that could inhibit pathogenic bacteria was unknown, while the results of our study used the microdilution method so that the smallest concentration of pigments that could inhibit bacteria could be known. In addition, the study used standard test bacteria, while in our study, the

Table 2. List of some microorganism that produce microbial melanin pigments

Microorganism	Objective	Main finding	Reference
P. stutzer	Melanin production from *P. stutzeri* isolated from red seaweed *Hypnea musciformis*	The marine *P. stutzeri* strain produces significant amounts of melanin of about 6·7 g l−1 without L-tyrosine supplementation in the sea-water production medium	(Ganesh Kumar *et al.* 2013)
Streptomyces cyaneus	Optimization of medium conditions using response surface methodology for melanin production by *S. cyaneus* and synthesis of copper oxide nanoparticles using gamma radiation	The unprecedented achievement was realized for melanin pigment production, (9.898 mg/ml) was obtained by optimized culture conditions. Also, 2.0% faba bean's seed peel maximized melanin (9.953 mg/ml) and hence super-yield (11.113 mg/ml) was produced by a stimulus from gamma irradiation (2.5 kGy)	(El-Batal *et al.* 2017)
Yarrowia lipolytica	Characterization of a nontoxic pyomelanin pigment produced by the yeast *Yarrowia lipolytica*	The ability of the yeast *Y. lipolytica* W29 to produce a high yield (0.5 mg/ml) of extracellular melanin was reported in a culture medium supplemented with L-tyrosine. The purified pigment was found to be embedded with antioxidant properties	(Tahar *et al.* 2019)
Amorphotheca resinae	Production and characterization of melanin pigments derived from *A. resinae*	*A. resinae* produced melanin in the peptone yeast extract glucose broth, reaching up to 4.5 g/L within 14 days. The structural properties of melanin are similar to eumelanin	(Oh *et al.* 2020)

bacteria used were MDR bacteria. In conclusion, our study shows that black pigment from *P. aeruginosa* PS1C marine isolate has antibacterial activity against MDR bacteria wound infections.

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