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Research Article

Isolation and Molecular Characterization of a Bacteria VSB3 from the Marine Environment and its Antibacterial Activities

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Abstract

The ocean's microbiological landscape forms a complex and integral part of its ecosystem, with burgeoning research shedding light on its significant metabolic capabilities and resilience within marine environments. Notably, pigments procured from sea-based bacteria have gained attention for their repository of naturally occurring compounds with advantageous pharmacological properties. These pigments are distinguished by their biological colouring features and are gaining recognition for their viability in diverse sectors, encompassing cosmetics, food production, textile manufacturing, print technology, and pharmaceutical science. The present research centres on the isolation and molecular characterization of a pigment-synthesizing bacterial isolate, named VSB3, sourced from marine samples extracted from the Bapatla and Yarada coastal regions. The unearthing of pigment-producing microbes from these oceanic areas, in conjunction with assessing their raw pigment extracts for antimicrobial effectiveness, presents an exciting frontier for the breakthroughs in antibiotic creation. From ten assorted marine bacterial candidates, eight showed capabilities of pigment biosynthesis; however, only a duo displayed considerable antimicrobial action. Confirmatory analyses of these isolates involved meticulous biochemical testing methods, while precise identification was done through analysis of the 16S rRNA gene sequence and subsequent phylogenetic evaluations. Eventually, VSB3 was taxonomically pinpointed as *Pseudomonas aeruginosa* following in-depth molecular scrutiny. Remarkably, the unrefined pigment solution yielded from VSB3 exhibited a strong antibacterial response against strains such as *Bacillus subtilis, Bacillus cereus*, and *Proteus vulgaris*. Manifestly, this inquiry accentuates the auspicious role of marine-derived bacterial pigments in fostering bioactive substances for industrial innovation and advancing medical treatment modalities.

Keywords: *Pseudomonas aeruginosa*; 16S rRNA; Phylogenetic Analysis; Antibacterial Activity; GC-MS Analysis; Bacterial Pigment; Marine Bacteria

Introduction

The marine environment encompasses more than 70% of the earth's surface and boasts an extensive array of biological diversity, contributing to over 90% of the biosphere and presenting a profusion of novel chemicals (Muller-Karger., *et al*. 2018). By virtue of its challenging conditions, the marine ecosystem and coastal regions foster a wealth of underexplored microorganisms. These tenacious organisms have adapted to endure extreme environmental factors such as intense pressure, frigid temperatures, elevated salinity, and limited nutrient availability (Somayaji., *et al*. 2022). As a result, the investigation of marine microorganisms has yielded the discovery of numerous bacteria endowed with biologically active compounds and diverse capabilities, including but not limited to bioremediation, textile dye biodegradation, and bioleaching (Torres, 2020). Marine microorganisms have risen to prominence as a valuable source of innovative microbial products, showcasing antibacterial, anticancer, antiviral, anticoagulant, anti-inflammatory, antioxidant, and cardioprotective properties (Karthikeyan., *et al*. 2022). Through the utilization of species-specific variability regions within the 16S rRNA gene sequence analysis, microbial identification can be achieved through phylogenetic distinctiveness, complementing phenotypic profiling. Over the past decade, there has been a surge

of interest in exploiting the potential of marine microorganisms as a rich reservoir of natural compounds. Our oceans harbour diverse microbial communities, constituting the largest habitat for microorganisms on our planet. Unveiling the hidden potential of marine microorganisms has led to the isolation of more than 300 structurally distinct bioactive natural compounds derived from both marine microbes and phytoplankton species, (Carroll., *et al*. 2021) alluding to their prominence in the quest to uncover novel bioactive agents. However, the exponential growth of aquaculture has encountered critical hurdles, primarily associated with disease outbreaks among aquatic animals and the contamination of water bodies, hindering the industry's expansion prospects. Aquatic species are susceptible to illness caused by viruses, parasites, and bacteria, with India emerging as the world's second-largest producer of fish, yielding approximately 9.06 million metric tons annually. India's significant contribution to the export market is evidenced by the production of nearly 700,000 tons of shrimp. The economic burden of aquatic animal diseases, particularly bacterial infections, is estimated to surpass INR 7,200 crores per year, signifying their detrimental impact on aquaculture as the leading cause of financial losses. *Vibrio, Aeromonas, Edwardsiella, Flavobacterium*, and Micrococcus stand as the predominant bacterial pathogens (Immanuel Suresh., *et al*. 2022). Progress in combating these challenges necessitates the use of antimicrobial substances in veterinary and medical applications.

In recent years, there has been a significant surge of interest in marine microorganisms as a promising reservoir of natural products. The world's oceans teem with diverse and yet uncharacterized microbial communities, representing the largest habitat for microorganisms on the planet. Marine microorganisms remain a vast, untapped source of biologically active natural compounds. In 2010 alone, over 300 structurally distinct bioactive natural compounds derived from marine microbes and phytoplankton species were identified, underscoring their potential as a rich resource for the discovery of bioactive natural products (Blunt., *et al*. 2016).

The rapid expansion of aquaculture has raised concerns regarding the health of aquatic organisms and pollution of water bodies, posing significant challenges to the industry's continued growth. Viruses, parasites, and bacteria are the primary culprits behind diseases affecting aquatic species. India, the second-largest fish producer globally, annually yields approximately 9.06 million metric tons of fish, with the production of nearly 700,000 tons of shrimp for export, making it one of the largest exporters. Bacterial infections, among other aquatic animal diseases, impose an estimated economic loss of 72 billion rupees on India's aquaculture sector. Notable bacterial pathogens responsible for these losses include Vibrio, Aeromonas, Edwardsiella, Flavobacterium, and Micrococcus (Wise., *et al*. 2023). The veterinary and medical sectors commonly employ antimicrobial agents to combat these pathogens.

Within aquaculture, antibiotics serve as potent agents for both prophylaxis and remediation of bacterial afflictions amongst cultivated species. However, the extended and improper application of such pharmaceuticals diminishes their efficacy, often culminating in the emergence of resistant pathogens that provoke exacerbated ailments. Residues of these antibiotics in aqua-derived consumables additionally constitute a tangible hazard to human health. Consequently, the innovation of novel antimicrobial entities for utilization within this sector attains critical importance. Compounds originating from biological sources offer advantages due to their rapid biodegradability, low toxicity profile, and inherent safety, rendering them ideal candidates for sourcing non-toxic antimicrobial ingredients suitable for aquatic contexts. Presently, marine-derived microorganisms stand as a prolific repository for the harvesting of antimicrobial agents that hold promise for aquaculture applications (Radjasa., *et al*. 2011). The discovery and accessibility of such biogenic substances are poised to revolutionize therapeutic approaches against bacterial disorders prevalent in aquaculture systems. Marine bacteria and fungi, with their extensive repertoire of structurally diverse and bioactive natural products, continue to be a pivotal informational resource in the ongoing search for efficacious antibacterial compounds targeting aquatic ailments. Among marine bacteria with significant biotechnological potential, Marine Pseudomonas species have garnered attention due to their ability to synthesize a plethora of substances including enzymes, bioactives, and biosurfactants [38]. Notably, Pseudomonas aeruginosa is distinguished by its pigment production capabilities. This Gram-negative bacterium exhibits proficiency in the synthesis of several soluble pigments such as brown pyomelanin, red pyorubin, yellow-green pyoveridin, and blue-green pyocyanin. Predominantly produced by this organism is pyocyanin – a water-soluble pigment belonging to the phenazine class – which it secretes extracellularly as a secondary metabolite [14]. Within medical literature, this pigment has been recognized for its diverse biological activities which

include induction of dose-responsive apoptosis in human pancreatic cancer cells (Panc-1), along with exhibiting broad-spectrum antimicrobial properties [15], and displaying antioxidant, antimalarial immunosuppressive, antiparasitic, and antibiofilm effects [16-19].

Materials and Methods

Bacterial cultures

The *Pseudomonas aeruginosa* VSB3 used in this investigation was isolated from a water sample taken from the Bapatla and Yarada beaches of India. Whereas Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *Bacillus subtilis* ATCC, *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC, *Enterococcus faecalis* ATCC 29212, *Proteus mirabilis* ATCC 25933, *Klebsiella pneumoniae* ATCC 700603, and *Proteus vulgaris* ATCC 49132, *Staphylococcus epidermidis* ATCC, *Salmonella enterica* ATCC, *Salmonella typhi* ATCC, were the twelve microbes used for the antimicrobial test.

Methods

Sampling site and Sample collection

Water samples are collected from the seashore and the middle of the sea.

The Beach sample is collected from Yarada 17°38'59.4"N 83°16'05.0"E and sea sample is collected from Bapatla 15°48'26.6"N 80°25'21.7"E.

Bacterial isolation methods

Quantification of viable bacterial populations was executed through the enumeration of colony-forming units (CFUs). This process was facilitated by culturing the microbes on Zobel's agar medium at 37°C for a duration of 24 hours utilizing the spread plate technique. Post incubation, selection of bacterial colonies was performed based on distinct macroscopic features such as morphological size, form, edge detailing, textural aspects, and pigmentation. These selected colonies were sub-cultured via repetitive streaking onto Zobel's agar surfaces or slanted growth mediums. For downstream analysis, these marine bacterial strains were preserved at 4°C.

Morphological assessment of bacteria

Observations regarding the morphological aspects such as chromatic variations, structural geometry, colonial boundary definition, elevational profiles, surface characteristics, and cellular arrangement of the isolated microorganisms were meticulously documented.

Gram-stain methodology

The morphological attributes of the microbial isolates were elucidated using the standard Gram-staining procedure prescribed in the literature reference [20].

Biochemical profiling

Biochemical activity assays adhering to established procedures were applied to all ten bacterial isolates to ascertain their enzymatic functions and aid in taxonomic classification as referenced in publication [21].

3.6 Optimization Experiments

For pH optimization: The medium's pH was carefully adjusted from 7.5 to 8.5 before introducing a bacterial isolate into the sterilized Zobel's medium—a systematic variation of incubation temperatures was employed thereafter. For temperature optimization: After establishing the optimal pH for incubation media, individual isolates were monitored under controlled thermal conditions ranging from 35 to 39°C to pinpoint the paramount temperature conducive to maximal secondary metabolite synthesis. Detection was carried out using an enzymatic immunoassay reader at an optical density of 600 nm (Epoch), with 200 µl aliquots dispensed into each well of a microtiter plate and absorbance readings taken bihourly over a period of 24 hours relative to a blank control.

Molecular Identification via 16S rRNA sequencing

Isolate VSB3 underwent molecular characterization by implementing sequencing techniques targeting its conserved 16S rRNA gene region—following deoxyribonucleic acid (DNA) extraction protocols established by Kimura M [22] and DNA confirmation via electrophoresis on a 1% agarose gel platform.

Polymerase Chain Reaction (PCR) amplification of the 16S rRNA gene fragment employed specific primers (16SrRNA-F and -R), consisting of an initial denaturation at 94°C lasting two minutes followed by thirty-five cycles comprising successive stages set at 94°C for 45 seconds, annealing at 55°C for a minute, extension at 72°C for another minute, finalized with an extension phase at 72°C for ten minutes. To get rid of impurities, the PCR amplicon was purified. Using the BDT v3.1 Cycle sequencing kit on an ABI 3730xl Genetic Analyzer, the forward and reverse DNA sequencing reaction of the PCR amplicon was performed using forward and reverse primers. The consensus sequence of the 16S rRNA gene was generated from forward and reverse sequence data using an aligner software.

Construction of phylogenetic tree

The 16S rRNA gene sequence of the isolate was used to carry out BLAST with the 'nr' database of the NCBI GenBank database. The multiple alignment software package Clustal W was used to select and align the top 10 sequences based on the maximum identity score. Distance matrix was generated, and the phylogenetic tree was constructed using MEGA 10.

Isolation of pigments

After being infused with ZoBell broth, the pigmented isolates were cultured for a week at 37°C. The cultures were centrifuged for 15 minutes at 4°C at 5000 rpm. If the colour is not visible in the supernatant, wash it with distilled water. Remove the supernatant, add 1 millilitre of ethanol to the pellet, and let it sit in a water bath for 60 minutes. After cooling it once again, centrifuge it for 15 minutes at 5000 rpm. Gather the supernatant and use Whatman's No. 1 filter paper to filter it [23].

Anti-bacterial activity

The antibacterial assay was conducted using the Agar well diffusion method, where the crude pigment extract of *Pseudomonas aeruginosa* was tested against a panel of bacterial strains, including Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *Bacillus subtilis* ATCC, *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC, *Enterococcus faecalis* ATCC 29212, *Proteus mirabilis* ATCC 25933, *Klebsiella pneumoniae* ATCC 700603, and *Proteus vulgaris* ATCC 49132. The bacterial strains were inoculated onto Nutrient Agar (NA) plates, and 100 µg/mL of the test bacterial culture was spread and plated on the NA plates. The wells were bored with a sterile well borer, to which the crude pigment extract of *Pseudomonas aeruginosa* was added. Ampicillin (1000 µg/mL) and uninoculated broth (1000 µg/mL) were used as positive controls in bacteria strains inoculated plates and as negative controls, respectively. The inoculated plates were then incubated at 37°C for 24 hours, and the clear zones formed were observed and the diameter was measured [24].

Results

Differential isolation and morphological analysis of marine bacterial strains

Utilizing the unique morphologies presented on Zobel's selective medium, a collection of 10 phenotypically diverse bacterial strains was elucidated from an oceanic sample. Morphological features and colonial characteristics of these bacterial isolates were meticulously documented (refer to Table 1). Each isolate exhibited specific physical and colonial morphology.

Phenotypic profiling of isolates via biochemical assays

Biochemical profiles of the isolates were elucidated employing an array of assays integral for taxonomical classification, encompassing methyl red assay, Voges-Proskauer assay, Indole production assay, citrate utilization assay, catalase enzyme assay, bacterial motility evaluation, haemolysis assay, and carbohydrate fermentation panels. Detailed outcomes are concisely tabulated in Table 3. Noteworthy variations among the isolates were observed in haemolysis, citrate metabolism, and catalase enzyme activity assays. The 10 isolated strains showcased divergent morphological and biochemical profiles alongside disparate colonial features, underscoring the structural and functional heterogeneity amongst them. Specifically, isolate VSB3 (visual representation in Figure 2), owing to its intriguing properties, was earmarked for comprehensive molecular characterization comprising phylogenetic delineation and 16S rRNA gene sequencing; its attributes were correlated with taxonomical data delineated from Bergey's Manual of Determinative Bacteriology.

Parametric optimization study

Plot 1 illustrates the experimental optimization data correlating temperature and pH preference over time. Temporal progression is delineated along the X-axis with temporal increments marked in

hours, while O.D. metrics are aligned on the Y-axis. Optimal growth conditions were determined to be at a temperature of 37 °C concurrent with a pH level of 7.7.

Genomic extraction procedures and molecular analysis of VSB3 strain

The genomic material from VSB3 was procured through the sarkosyl extraction protocol with subsequent validation achieved via electrophoresis on a 1% agarose gel matrix, where a singular band indicative of high-molecular-weight DNA was discerned (Figure 4). The pivotal role of molecular methodologies in definitive identification served as impetus for such analyses on VSB3's DNA samples. The significance of 16S rRNA gene sequences in clarifying bacterial taxonomy necessitates this level of investigation for accurate species-level resolution. Following amplification by universally conserved primers targeting regions flanking the 16S rRNA gene (16S rRNA-F and 16S rRNA-R), a conspicuous amplicon approximating 1500 base pairs was detected post-gel electrophoretic separation (Figure 4). Purification ensued to eliminate non-specific products or potential contaminants prior to sequencing reactions conducted using both forward and reverse primers; these processes were

Phylogenetic analysis

The 16S rRNA gene sequences of the isolate were subjected to the BLAST online tool in the database of NCBI GenBank. Phylogenetic analysis was performed based on maximum identity scores; the first ten sequences were selected and aligned using the multiple alignment software program Clustal W. The molecular and phylogenetic analysis of 16SrRNA gene sequences revealed the isolate, VSB3 showed identity to Pseudomonas aeruginosa (99%). The distance matrix was generated, and the phylogenetic tree was constructed using MEGA 11 (Figure 5). The evolutionary history was inferred by using the maximum likelihood method and the Tamura-Nei model based on the Kimura 2-parameter model. Evolutionary analyses were conducted in MEGA11. The evolutionary history of the species under study was assumed to be represented by the bootstrap consensus tree that was derived from 500 replicates.

Anti-bacterial activity

The results of the antimicrobial experiment showed that the crude pigment extract of *P. aeruginosa* VSB3 has antibacterial activity, as evidenced by the creation of inhibition zones, which inhibited the growth of four tested bacteria (Figure 6). The increased concentration of crude pigment extract that was employed resulted in a bigger inhibition zone. Both Gram-positive and Gram-negative bacteria were actively combated by this extract's antibacterial properties. At a dosage of 1000 µg/mL, the antibacterial activity of all the targeted microorganisms was seen.

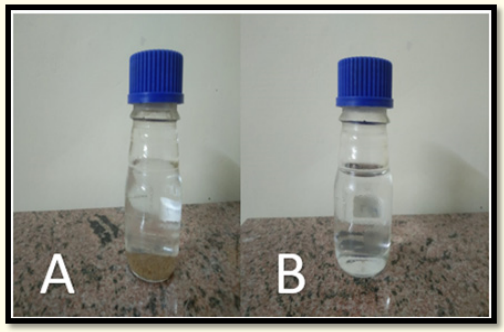


Figure 1: Water samples collected from A. Yarada Beach sample. B. Bapatla sea sample.

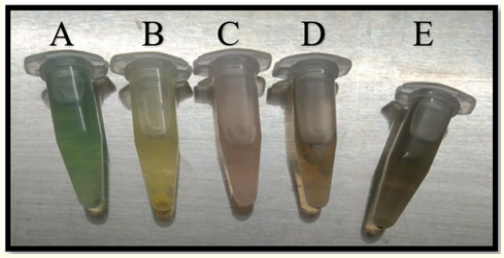


Figure 2: Pigments isolated from A) V1 B) S1 C) S2 D) V2 E) VSB3.

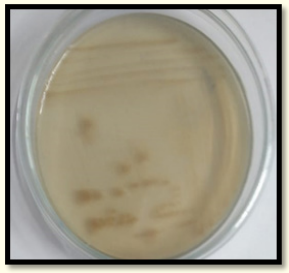


Figure 3: VSB3.

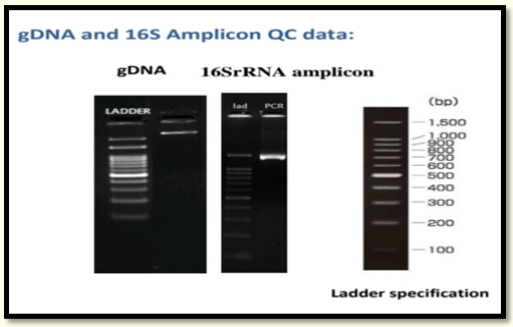


Figure 4: Amplification of 16S rRNA gene of VSB3 isolate.

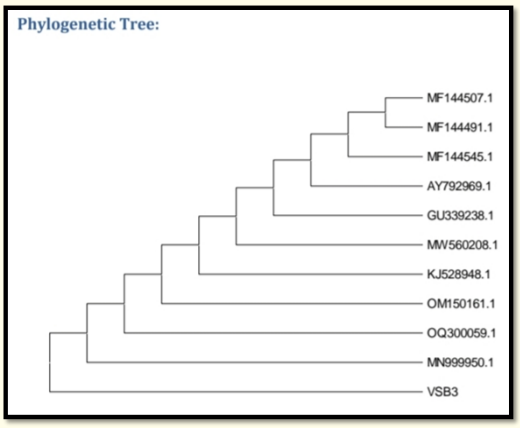


Figure 5: By applying the maximum likelihood approach, the 16S rRNA sequence is used to build the tree.

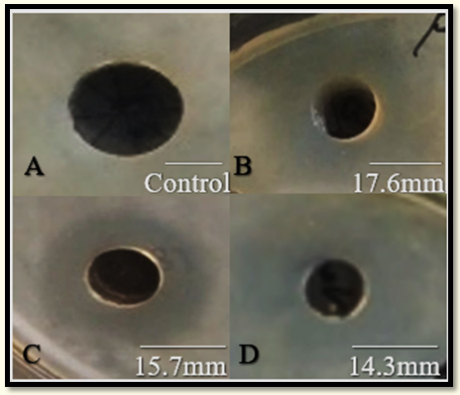


Figure 6: Antibacterial activity of *P. aeruginosa* VSB3-derived crude pigment extract (1000µg/mL) against A. Control B. *P. vulgaris* C. *B. subtilis D. B. cereus.*

Discussion

The study of antagonistic interactions among marine microbes in oceanic environments is a subject of significant scientific interest due to the potential discovery of organisms proficient in the production of secondary metabolites. Historically, the isolation and exploration for bacteria capable of synthesizing these compounds have received insufficient attention. Recent investigations have uncovered that a cohort of eight marine bacteria, symbiotic with marine organisms, possess capabilities to inhibit the growth

Marine isolates	Shape	Margin	Elevation	Size	Texture	Appearance	Pigmentation	Optical property
V1	Circular	Entire	Raised	Small	Smooth	Shiny	Yellow	Opaque
V2	Circular	Entire	Raised	Small	Smooth	Shiny	Pale orange	Opaque
VSB3	Irregular	Curled	Flat	Large	Smooth	Shiny	Green	Transparent
V4	Irregular	Curled	Flat	Large	Smooth	Shiny	Olive green	Transparent
V5	Irregular	Entire	Raised	Large	Smooth	Shiny	Orange	Opaque
V6	Irregular	Curled	Raised	Large	Rough	Dull	Non-pigmented	Opaque
S1	Circular	Entire	Raised	Small	Smooth	Shiny	Honey yellow	Opaque
S2	Circular	Entire	Raised	Small	Smooth	Shiny	Magenta	Opaque
S3	Circular	Entire	Raised	Small	Smooth	Shiny	Pale orange	Opaque

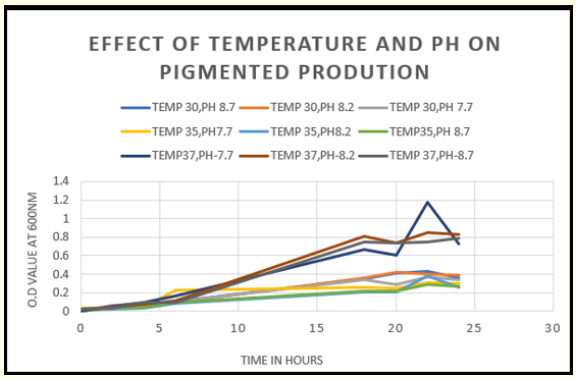
Table 1*:* Morphological characteristics of bacterial strains that have been isolated.

Marine isolates	Morphology
V1	Gram-positive, Staphylococci, non-motile.
V2	Gram-negative, cocci, non-motile.
VSB3	Gram-negative, bacilli, motile.
V4	Gram-negative, bacilli, motile.
V5	Gram-negative, bacilli, non-motile.
V6	Gram-negative, streptobacilli, non-motile.
S1	Gram-positive, cocci, non-motile.
S2	Gram-negative, cocci, non-motile.
S3	Gram-negative, cocci, non-motile.

Table 2: Gram nature of the isolates.

Biochemical test	V1	V2	VSB3	V4	S1	S2
Catalase test	+	+	+	+	+	+
Indole test	-	-	-	-	-	-
Methyl red test	-	-	-	-	-	+
Voges Proskauer test	-	-	-	-	-	+
Citrate utilisation	-	-	+	+	-	+
Glucose fermentation test	-	-	-	-	-	-
Lactose fermentation test	-	-	-	-	-	-

Table 3: Biochemical characterisation of isolated bacterial strain.



Graph 1: Optimization study of the bacterial isolate VSB3.

of pathogenic microbes. Exhibit 6 presents the inhibitory effects displayed by marine bacterial isolate VSB3 on Proteus vulgaris, a Gram-negative pathogen. This suppressive effect was observed to be more pronounced when compared with that against Gram-positive species such as Bacillus subtilis and Bacillus cereus. The categorization of inhibitory zones revealed that diameters less than 16.0 mm represent robust inhibition, diameters between 11 and 16 mm denote moderate inhibition, those from 7 to 11 mm indicate weak inhibition, and those under 7 mm signify a lack of activity.

Subsequent to a 48-hour incubation period, the bacterial strain *P. aeruginosa* VSB3—sourced from a marine water sample during this investigation—produced a pigment yielding a blue-green hue on Zobel's agar. This pigment's crude extract exhibited extensive antibacterial efficacy against a spectrum of Gram-positive and Gram-negative bacteria, including but not limited to Bacillus subtilis ATCC, Bacillus cereus ATCC, and Proteus vulgaris ATCC 49132. The pigment extract manifested the most significant zone of clear inhibition against Gram-negative bacteria while displaying peak antibacterial potency towards *P. vulgaris* at a concentration of 1000 µg/ml. Differences in cell wall architecture between Gram-positive and Gram-negative bacteria likely contribute to disparities in susceptibility to antibacterial agents; specifically, the augmented lipopolysaccharide composition in Gram-negative bacteria may offer them increased resistance. Contrastingly, Gram-positive bacteria

lack this protective outer layer, rendering them more vulnerable to antimicrobial agents.

The present research delineates the bacteriostatic function exercised by the crude extract of *P. aeruginosa* VSB3's pigment on these pathogens. Chemical analyses—namely gas chromatography-mass spectrometry (GC-MS) previously undertaken by scholars—attributed fatty acids as pivotal constituents within this extract. Predominant compounds such as trans-2-decenoic acid and hexadecenoic acid have been implicated for their notable antimicrobial, antioxidant, and antineoplastic properties. This isolate is a possible source of biologically active substances and needs to be further studied, particularly its prospect for biomedical applications.

Conclusion

The aim of the current inquisition has been to identify and characterize the bacterial isolate known as VSB3, which was found in the maritime waters of Bapatla and Yarada. The strain was described using biochemical testing and 16S rRNA molecular sequencing, followed by phylogenetic analysis. According to the findings of the molecular characterization, *Pseudomonas aeruginosa* and the isolate VSB3 are 99% analogous. The test bacteria, which often spawn nosocomial infections, was successfully stopped from growing by the isolate. Overall results in the present study suggest that isolating VSB3 might be exploited in the development of new treatments for infections. To bolster the molecular mechanism of pathogen suppression by the isolate VSB3, more research is required to design novel treatments and antibacterial methods. The findings of this study have broader counteraccusations for the field of drugs and could potentially pave the way for new approaches to precluding and treating contagious diseases.

Conflict of Interest

The authors have no conflict of Interests.

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