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**Original Research Article**

# Sea Sand Inhibits Growths of Extremely Drug Resistant Bacteria

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**Abstract:** Numerous reports are showing the presence of both pathogenic and non-pathogenic microbes in sea sand, and sea sand specified to conserve drug resistance genes. However, there is not any study to realize whether drug-resistant bacteria can thrive in sea sand or not. In this investigation, we observed events of some extremely drug-resistant bacteria in sea sand. Our study pointed to diminished growths of these extremely drug-resistant bacteria in sea sand and surges release of bacterial natural pigments, which additionally could check microbial growths. Therefore, a negative feedback system de novo is dynamic in sea beaches to limit bacterial residents.

**Keywords:** Sea sand, Sea beach, drug resistant bacteria, pyocyanin.



## INTRODUCTION

Beaches are crucial for recreation and tourism, with beachgoers prioritizing cleanliness. Although a decline was documented during the COVID-19 pandemic, 21st-century tourism remains a multifaceted and substantial activity [1].

In 2019, tourism contributed to 10.3% of the global GDP, with coastal and maritime tourism emerging as the predominant sector [2]. The extensive, pristine sandy beaches along coastlines (Fig.1) have evolved into crucial economic hubs for tourism, estimating a contribution of USD 1.9 trillion in 2021 [3,4]. However, apparent cleanliness does not guarantee freedom from health hazards, as sand can harbour microbes. Estimating the microbiological quality of beach sand and seawater is essential for assessing beach safety.

Recent research indicates that direct exposure to beach sand poses a risk of infectious diseases, especially in children. Sand contains various microorganisms, including bacteria, viruses, protozoa, helminths, and fungi, originating from the environment, animals, or humans. Pathogenic bacteria found in beach sands include *Vibrio vulnificus*, *Salmonella*, *Campylobacter*, *Pseudomonas aeruginosa*, *and Staphylococcus aureus* [5-7].

**Fig 1: Sea beach with a consortium of sand, water, wind, microbes, human beings and animals**

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Coastal water quality is influenced by inorganic macronutrients, bearing phytoplankton abundance and biodiversity. Nutrient inputs can lead to eutrophication, measured by chlorophyll A concentration. Changes in water colour and transparency, along with phytoplankton proliferation, can alter seawater appearance, affecting aesthetics but posing no health risk unless harmful algal species are present [8].

The quality of seawater is a crucial concern, and various studies have consistently identified elevated levels of faecal indicator bacteria in sediments. Consequently, sediments serve as substantial reservoirs for bacteria, and their resuspension, associated with diverse natural processes and human activities, emerges as a notable mechanism for disseminating pathogens. This process holds significance as it can significantly impact assessments of seawater quality mandated by current regulations.

Nevertheless, there is currently no global regulation establishing permissible limits for these microorganisms, similar to those defined for the water column. Various researchers emphasize the necessity for studies to elucidate the influence of sediment-bound bacteria on water quality and the well-being of beachgoers. [10-12] These studies aim to establish standardized methods for sampling and quantification. Results, such as those presented in this study, aspire to stimulate discussions within the scientific community and environmental authorities across diverse nations.

Microorganisms bound to sediment exhibit prolonged viability, enduring for months, a contrast to those existing planktonically in the water column with a survival span of only a few days [13]. Sediments provide favourable conditions for bacteria, offering attachment sites conducive to biofilm formation, a source of organic substances and nutrients, and protection against environmental stressors like sunlight and protozoan grazing [14].

Numerous investigations have noted that bacteria in aquatic settings are frequently associated with fine, cohesive sediment particles (<60 µm) [15]. Attraction to sediments initially occurs through London–van der Waals forces, with subsequent utilization of extracellular polymers for robust and enduring adhesion once in close proximity to the surface [16].

However, the resilience of sediment-bound microorganisms is influenced by various factors, encompassing biological elements (e.g., predation and competition), environmental conditions (e.g., particle size, temperature, humidity, nutrients, and sunlight), and physical forces [17, 18].

**THE RISING CONCERN FOR ANTIMICROBIAL RESISTANCE STRAINS:**

The global challenge of antimicrobial resistance demands urgent attention and effective solutions with regard to both nosocomial and community-acquired infections. This concern extends beyond developed nations, where comprehensive policies govern antimicrobial drug usage, and resources are available to assess resistance levels [19]. Indeed, the antimicrobial resistance has recently been identified as one of the three most important problems facing human health by World Health Organization (WHO) [20]. The utmost habitual and significant MDR pathogens have been incorporated within the acronym "ESKAPE," which stands for *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp [21].

When antimicrobials are employed, bacteria invariably develop resistance mechanisms, either through spontaneous mutations or the acquisition of genes from other bacteria. The latter can transpire through processes such as transduction, facilitated by bacteriophages, or conjugation, entailing direct cell-to-cell contact with the transfer of plasmids or transposons. Additionally, transformation, characterized by the uptake of free DNA released during bacterial lysis, serves as another avenue for the development of resistance [22, 23].

Here, we employed an approach to monitor the growth or inhibition of the drug resistant bacteria (MDR strain) like *E coli, Pseudomonas aeruginosa and Klebsiella pneumoniae* in extra pure sea sand collected from sea beach and processed appropriately to make it extra pure. The aims of this experiment are to show that sand sediments act as a possible reservoir or inhibitor of such drug resistant microorganisms which helps them to either survive and thrive better or inhibiting the growth of these MDR bacteria compared to their normal growth, in addition, evidence relationships between microorganism counts and environmental and human factors.

## MATERIALS AND METHODS

**PROCURATION OF SAND USED –** The sand here used in the experiment is acquired from HiMedia (RM3062 Sea sand, Extra pure, Mol. Wt. :60.08, CAS No. :14808-60-7). When examined under microscope (40x) the specks were rough enough to grasp microbes (Fig.2).

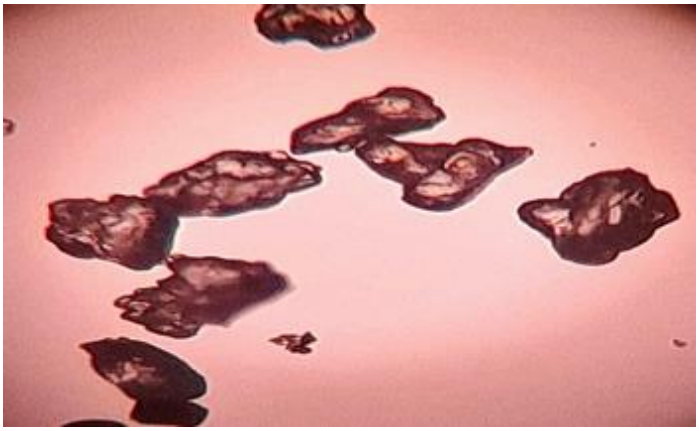

**Fig. 2: Sea Sand particles under microscope**

**MICROORGANISM UNDER STUDY:**

The experiment comprised three distinct setups, each focusing on Multi-Drug Resistant bacteria, namely *E. coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. These bacterial strains were isolated from urine, gallbladder aspirates, and pus samples respectively of critical care patients admitted in a tertiary care hospital of Kolkata, India (Peerless Hospitex Hospital and Research Center Limited, Pancha Sayar, Kolkata, West Bengal 700094). The names of the patients were kept anonymous as per guidelines of the Ethical Committee of the Hospital. The fundamental characteristics of each bacterium are detailed in the tables (Table 1-3).

**Table 1: The antibiogram of *Escherichia coli*, it was a pan-resistant bacterium interpreted by Vitek 2 compact automated system (bio Merieux SA, France)**

| **Organism: *Escherichia coli* (MDRO)** | | |
|---|---|---|
| **Antimicrobial agents** | **MIC (µg/ml)** | **Interpretation R-Resistant** |
| Amoxicillin/Clavulanic Acid | >=32 | R |
| Piperacillin/Tazobactam | >=128 | R |
| Cefuroxime | >=64 | R |
| Cefuroxime Axetil | >=64 | R |
| +Cefixime | | R |
| Ceftriaxone | >=64 | R |
| Cefoperazone/Sulbactam | >=64 | R |
| Cefepime | >=32 | R |
| +Doripenem | | R |
| Ertapenem | >=8 | R |
| Imipenem | 8 | R |
| Meropenem | 8 | R |
| Amikacin | >=64 | R |
| Gentamicin | >=16 | R |
| Ciprofloxacin | >=4 | R |
| +Levofloxacin | | R |
| Trimethoprim/Sulfamethoxazole | >=320 | R |

**Table 2: The antibiogram of *Pseudomonas aeruginosa*, except Cefoperazone/Sulbactam it was resistant to other antibiotics as interpreted by Vitek 2 compact automated system (bioMerieux SA, France)**

| **Organism: *Pseudomonas aeruginoda* (MDRO)** | | |
|---|---|---|
| **Antimicrobial** | **MIC (µg/ml)** | **Interpretation R-Resistant I-Intermediate** |
| Piperacillin/Tazobactam | >=128 | R |
| Ceftazidime | >=64 | R |
| Cefoperazone/Sulbactam | 32 | I |
| Cefepime | >=32 | R |
| Aztreonam | >=64 | R |
| +Doripenem | >=16 | R |

| **Organism: *Pseudomonas aeruginoda* (MDRO)** | | |
|---|---|---|
| **Antimicrobial** | **MIC (µg/ml)** | **Interpretation R-Resistant I-Intermediate** |
| Imipenem | >=16 | R |
| Meropenem | >=16 | R |
| Amikacin | >=64 | R |
| Gentamicin | >=16 | R |
| Ciprofloxacin | >=4 | R |
| Levofloxacin | >=8 | R |
| Minocycline | >=8 | R |
| Tigecycline | >=8 | R |
| Fosfomycin | >=256 | R |
| Colistin | >=16 | R |
| +Polymyxin B | | R |

**Table3: The antibiogram of *Klebsiella pneumoniae subspecies pneumoniae*, it was a pan-resistant bacterium interpreted by Vitek 2 compact automated system (bioMerieux SA, France)**

| **Organism: *Klebsiella pneumoniae ssp pneumoniae* (MDRO)** | | |
|---|---|---|
| **Antimicrobial** | **MIC (µg/ml)** | **Interpretation R-Resistant** |
| Amoxicillin/Clavulanic Acid | >=32 | R |
| Piperacillin/Tazobactam | >=128 | R |
| Cefuroxime | >=64 | R |
| Cefuroxime Axetil | >=64 | R |
| +Cefixime | | R |
| Ceftriaxone | >=64 | R |
| Cefoperazone/Sulbactam | >=64 | R |
| Cefepime | >=32 | R |
| +Doripenem | | R |
| Ertapenem | >=8 | R |
| Imipenem | >=16 | R |
| Meropenem | >=16 | R |
| Amikacin | 32 | R |
| Gentamicin | >=16 | R |
| Ciprofloxacin | >=4 | R |
| +Levofloxacin | | R |
| Trimethoprim/ Sulfamethoxazole | >=320 | R |

**Preparation of sample for the experiment:-** Three test tubes for each of the bacterial strain were taken, which were classified and were composed of:

**SETUP 1 - Nutrient Broth + Bacteria (either of *E coli, Pseudomonas* or *Klebsiella*)**

In this study, 2 mL of sterile Nutrient Broth was dispensed into each of three separate sterilized test tubes. Subsequently, the three test tubes were inoculated with distinct bacterial strains, resulting in the creation of three unique sets of growth suspensions for each bacterial strain. Then these test tubes are allowed to incubate at $37^{O}$C in an incubator for 1-2 hrs. Here the McFarland standard was kept at 0.5 McFarland standards serve as a benchmark for adjusting the turbidity of bacterial suspensions, ensuring a standardized bacterial count for microbial testing. This is particularly crucial in antibiotic susceptibility testing, where the minimum inhibitory concentration is measured, a common practice in medical microbiology and research. Deviations in suspension density may lead to inaccurate results, presenting either false resistance or false susceptibility to specific antimicrobial agents. This methodology was employed to prepare and standardize growth suspensions for subsequent analysis of the respective bacterial strains. Densichek was used to measure the optical density of microbial suspension. It provides a user-friendly experience featuring a touchscreen interface that displays McFarland values. Users can easily select the card type on the touchscreen, and the expected McFarland ranges are promptly presented for convenient reference.

**SETUP 2 – (Control setup – Nutrient Broth + Sand)**

In the control experiment, a standardized quantity of 1 gram of sand was measured using a precision weight machine. This measured amount of sand was then introduced into individual test tubes and autoclaved. Subsequently, 1 mL of sterile nutrient broth was added to each test tube, resulting in the establishment of three

distinct control setups for each bacterial strain. This meticulous procedure ensured the consistency and reproducibility of the control conditions across the experimental set.

**SETUP- 3 (Test setup – Sand+ Nutrient Broth + Bacteria)**

In the initial step, 1 gram of precisely measured sand was introduced into each of the three test tubes and autoclaved. Subsequently, 1 mL of the culture suspension from Setup 1 for each respective bacterial strain was pipetted into these test tubes. This sequential procedure was employed to create standardized test setups for the different bacterial strains under investigation. The meticulous implementation of these steps ensured consistency and reliability in the preparation of test conditions for subsequent analyses.

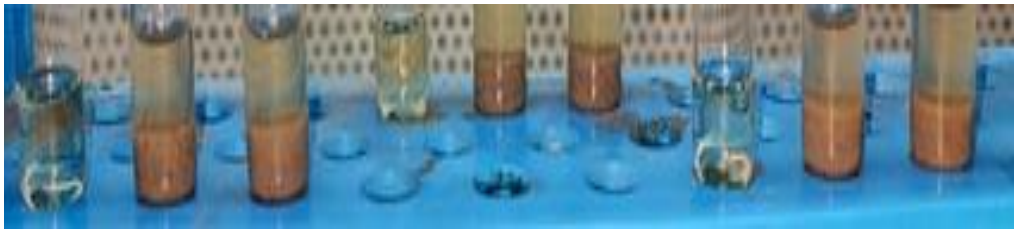

**Fig.3 Setup 1, setup 2 and setup 3 for *E. coli, Pseudomonas,* and *Klebsiella***

The experiment was executed by preparing samples and subsequently incubating the test tubes for a duration of 24 hours in a controlled incubator environment at $37^{O}$C. Following the incubation period, suspensions were extracted from the test tubes, and their optical density was quantified at a wavelength of 600 nm using a spectrophotometer. For better understanding the above experiment was conducted twice and we obtained two sets of observations which are given below. We did not repeat the test further, as the interpretations were similar in the two experiments due to potential risk of work with pan-resistant bacteria.

**OBSERVATIONS (OD values) :**

| **OBSERVATION 1:** | | | | |
|---|---|---|---|---|
| **Microorganism** | **Nutrient broth + Microbe** | **Control (Nutrient broth + Sand)** | **Test (Sand + Nutrient broth + Microbe)** | **Variation (test – control)** |
| *Escherichia coli* | 0.3079 | 0.0548 | 0.3295 | 0.2747 (Inhibited) |
| *Klebsiella pneumoniae* | 0.3842 | 0.0580 | 0.2814 | 0.2234 (Inhibited) |
| *Pseudomonas aeruginosa* | 0.2867 | 0.0581 | 0.5776 | 0.5195 (OD increased) |

| **OBSERVATION 2:** | | | | |
|---|---|---|---|---|
| **Microorganism** | **Nutrient broth + Microbe** | **Control (Nutrient broth + Sand)** | **Test (Sand + Nutrient broth + Microbe)** | **Variation (test – control)** |
| *Escherichia coli* | 0.2934 | 0.0443 | 0.1912 | 0.1469 (Inhibited) |
| *Klebsiella pneumoniae* | 0.2177 | 0.0448 | 0.1597 | 0.1149 (Inhibited) |
| *Pseudomonas aeruginosa* | 0.1550 | 0.0447 | 0.2897 | 0.2450 (OD increased) |

A notable reduction in growth was evident in the test setup compared to Setup 1 with *Escherichia coli* and *Klebsiella pneumoniae*. Substantial variations in bacterial growth were observed between these two setups. This observation underscores the significance of experimental conditions and highlights potential factors influencing microbial proliferation. The findings contribute valuable insights into the dynamics of bacterial growth under different experimental parameters, emphasizing the need for further investigation into the underlying mechanisms governing these variations. These results are pivotal in advancing our understanding of microbial behaviour in diverse experimental contexts.

**PSEUDOMONAS PIGMENT FORMATION TEST:**

As *Pseudomonas aeruginosa* produces diffusible pigment, the increased OD observed in the experiment may be due to the pigment formation. Thus, we compared bacterial growth and pigment formation of *Pseudomonas* by inoculating one loop-full of the growth each from nutrient broth culture tube and the test culture tube on Mueller Hinton medium, incubated overnight in incubator at $37^{O}$C. We observed mild inhibition of bacterial growth in test culture and a distinct increase of pigment formation (Fig. 4) indicating increased OD in this set was due to formation of the pigment.

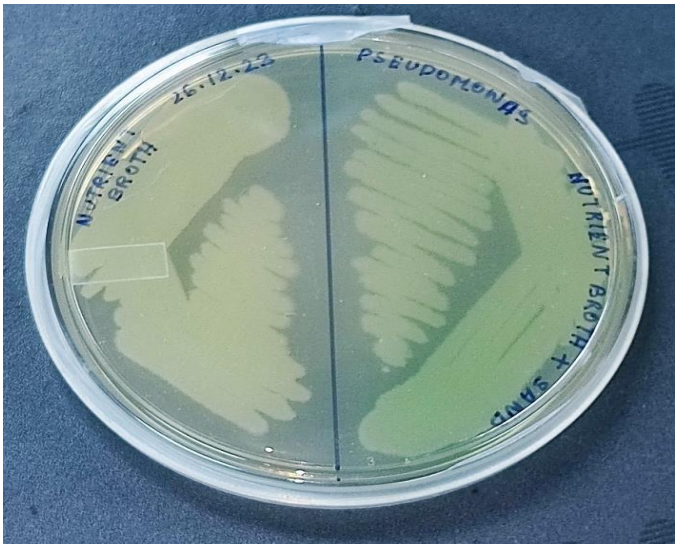


**Fig.4: Increased pigment formation of *Pseudomonas aeruginosa* in sea sand**

## DISCUSSION

The sea beach is a complex consortium of sand, water, wind, beachgoers, animals, and microorganisms. It is important to note that beachgoers spend more time on sand than in water as observed by WHO [24]. Although studies on microorganisms of sea sand date back to 1960, only recently, its importance to human health has been apprised [25] and in 2020 WHO indicated that, nearshore microbial exposure is important for health-related conditions [26]. Minerals and water of beach sand can harbour a wide variety of microorganisms with the formation of biofilms [27, 28], thereby the microbial community in sea sand is unique and not representative of the seawater which passes over it. Although sand particle size is different in different beaches it is not related to microbial diversity [29], however, sand roughness may lead to more microbial adherence [30]. Some microbial variation also occurs in the sand of the swash zone and the sand of the supratidal zone.

*E. coli* is an indicator bacteria of sewage pollution and some studies indicated that beach sand may hold a significant quantity of indicator bacteria [31, 32]. It may be due to passive contamination or due to active growth of the bacteria in the sand. In the USA, if the beach water *E. coli* count surpasses 235 cfu/100 ml then the beach is closed for swimming. There is no such standard boundary in beach sand and although it is an essential concern, it is still unsolved. Our study indicates that the count of *E. coli* in sand primarily depends on passive contamination and not on the active growth of *E. coli* in it.

Survival of bacteria in sand depends on diverse genetic factors mainly related to metabolic enzymes and transport proteins [33]. However, *E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* showed linear decay in freshwater sediment experiments[34] which corroborates with our finding indicating there is no active growth of bacteria in the sand.

In this study, we used mainly pan-resistant bacteria. Many of the drug-resistant genes are originated from the environment [35]. About $10^{30}$ bacterial and archaeal cells are present on Earth with a large reservoir of drug-resistant genes [36]. Although *Klebsiella pnemoniae* is commonly associated with antibiotic resistance and is present in diverse environments, we have little knowledge about it in marine environments and particularly in beach sand. At present, the environment is recognised as an important source of antibiotic resistance [37].

There are more than 200 species and subspecies of *Pseudomonas* [38]. Among them, the known human and animal pathogen *Pseudomonas aeruginosa* is the type species of this Genus. *Pseudomonas aeruginosa* is found in beach sand more frequently than water as observed in studies of Israel [39] and Portugal [40].

One of the reasons for decreased bacteria in sea sand is the photo-catalytic activities of sea sand. Silica is mainly obtained from beach sand [41]. Like TiO2, SiO2 has also some photocatalytic activity causing damage to bacterial cells [42].

Pseudomonas pigment is a secondary metabolite for their protection and persistence [43]. Natural pigments are biodegradable [44]. Thus, pyocyanin- the blue phenazine pigment produced by *Pseudomonas* is used in different types of industries [44-46]. It is also used as a biosensor, adjuvant, antibacterial, antifungal, antioxidant and anticancer substance in medicine, and also applied in the fields of agriculture and aquaculture.

The *aro* pathway is important for the synthesis of pyocyanin in which chorismic acid is produced from shikimic acid [47]. Phenazine-specific methyl

transferase and flavin-dependent monooxygenase genes regulate pyocyanin formation as phenazine-1-carboxylic acid is formed from chorismic acid. 5-methyl phenazine1-carboxylic acid betaine is formed from Phenazine1-carboxylic acid by Phenazine-specific methyl transferase; then 5-methyl phenazine1-carboxylic acid betaine is catalysed by flavin-dependent monooxygenase to pyocyanin [48]. Acyl-homoserine lactone (AHL) is a good inducer of pyocyanin in *Pseudomonas* [49]. Nutrients particularly nitrogen sources, temperature, pH and aeration are important factors for pyocyanin formation [50]. Sucrose, glycerol, glucose, cetrimide, mannitol, and maltose, as carbon sources increase pyocyanin formation [50, 51]. Different metals iron, zinc etc. also increases pigment. Our study also corroborates studies on silicon-utilizing organisms where these common organisms are not listed [52, 53] for utilization of silicon.

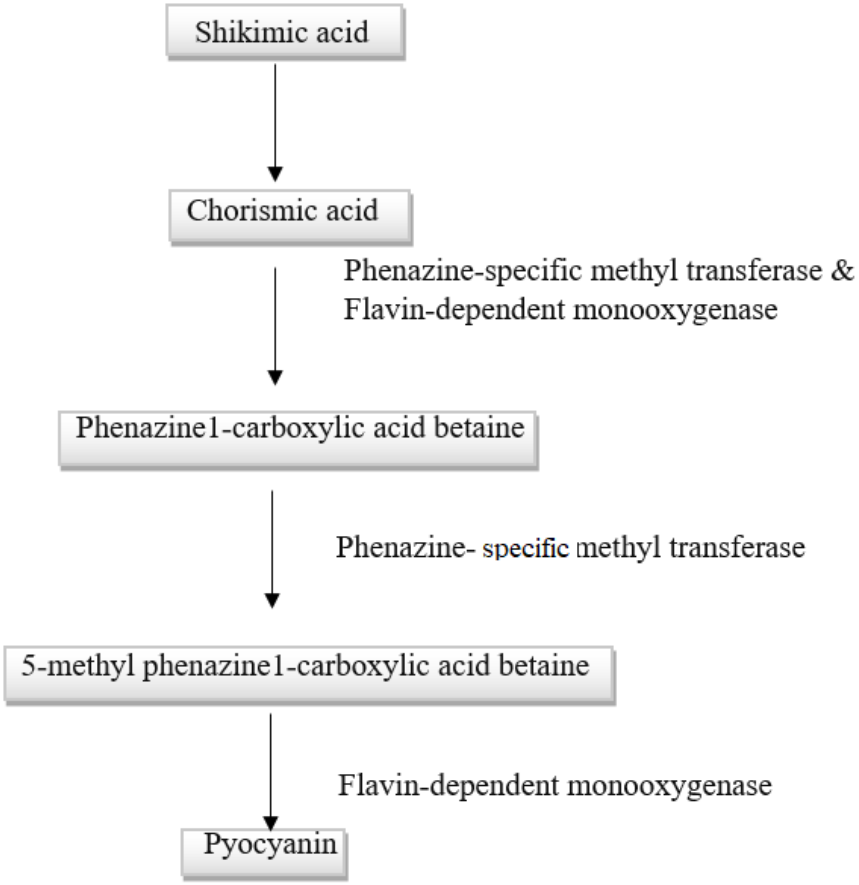


**Fig. 5: Formation of Pyocyanin from Shikimic Acid**

## CONCLUSION

In conclusion, the sea beach environment is a complex ecosystem comprising sand, water, wind, beachgoers, animals, and a diverse microbial community. Recent recognition of the importance of nearshore microbial exposure to human health emphasizes the need to understand the dynamics of microbial populations in beach sand. While the microbial community in sea sand forms unique biofilms, it differs significantly from the microbial composition in seawater. The presence of indicator bacteria such as *E. coli* in beach sand raises concerns, with our study indicating that their counts primarily result from passive contamination rather than active growth. Genetic factors play a crucial role in the survival of bacteria in sand, and our findings align with previous research, suggesting linear decay rather than active growth of bacteria in freshwater sediment experiments. Drug-resistant bacteria, including *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, are present in beach sand, highlighting the beach environment as a potential source of antibiotic resistance genes.

The study sheds light on the photo-catalytic activities of sea sand, particularly the damage caused to bacterial cells by silica, a major component of beach sand. *Pseudomonas aeruginosa*, frequently found in beach sand, produces the secondary metabolite pyocyanin through the *aro* pathway. Pyocyanin, a blue phenazine pigment, has diverse applications in various industries, medicine, agriculture, and aquaculture. The study also highlights the factors influencing pyocyanin formation, including AHL inducers, nutrient availability, temperature, pH, aeration, and carbon sources.

Overall, the research provides valuable insights into the microbial ecology of beach sand, emphasizing its role as a reservoir of diverse microorganisms and antibiotic resistance genes. Understanding the dynamics of microbial communities in beach environments is crucial for addressing public health concerns and optimizing the utilization of natural resources, such as pyocyanin, derived from these ecosystems.

**Conflict of Interest:** The authors declare no conflict of interest.

**Author's Contribution**: Dr. Satadal Das designed the study procedure and procured the sea sand. Mr. Arijit Halder and Ms Nikita Parui carried out the experiment, analysed the data and wrote the manuscript. Dr. Satadal Das and other Authors reviewed and edited the manuscript.

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