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STUDY OF BIOACTIVE COMPOUNDS FROM POTENTIAL MARINE MICROORGANISMS

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ABSTRACT

Research on marine bacteria has highlighted the tremendous potential of microorganisms as a source of new bioactive secondary metabolites and it is reported that most of the marine bacterial pigments exhibit antimicrobial activity. Despite of great difficulty in isolating and harvesting marine bacteria, their metabolites are increasingly attractive to science because of their broad-ranging pharmacological activities especially those with unique colour pigments. Bacterial pigments offer promising avenues for various applications in industries like food, pharmaceuticals, cosmetics, textiles due to their biodegradability and higher compatibility with the environment. Marine *Actinomycetes* survive in extreme conditions such as high salinity, low temperature and are able to produce bioactive secondary metabolites which are stable at extreme conditions and show antiparasitic, antioxidant, antibacterial, antifungal and antitumor activities. *Actinomycetes* and pigmented organisms were isolated from marine water sample. The extracted bacterial pigments had antibacterial activity. The antibiotic sensitivity of the pigment was further checked. DNA extraction and PCR analysis was performed. Morphological studies and gene sequencing confirmed the presence of bacterial strain *Salinococcus roseus and Micrococcus endophyticus* and *Actinomycetes*: *Streptomyces werraensis.* Further studies could prove antioxidant and anti-cancerous properties of the pigment.

KEYWORDS: Metabolites, pigment, antibacterial activity, antibiotic sensitivity.

INTRODUCTION

Marine biology is a discipline which strives to understand and solve the problems regarding the sustainable exploration of marine life for the benefit of human health through the cooperation between scientists working in marine biology, molecular biology, Microbiology, Chemistry and different disciplines. Several success stories of the applications of molecular techniques in the field of marine biology are helping in further research in this area.[1]

Marine extremophiles can be divided on the basis of habitat into psychrophiles (living at low temperatures), halophiles (living at high salinity), and barophiles (living under high pressure) [2].Marine microorganisms are excellent source for antimicrobial compounds.[3] Marine Actinomycetes survive in extreme conditions such as high salinity, low temperature and are able to produce bioactive secondary metabolites which are stable at these conditions. (R. Solanki et al, 2008).[4]

The microbial pigments are of great interest due to the applicability of the pigments produced by them[5] and the availability of cultivation technology.[6][7] Marine bacteria, however, are attractive to researchers because they can potentially produce compounds with unique biological properties.[8]

Both natural pigments and synthetic dyes have been extensively used in various fields of everyday life such as foods/feeds, textile, paper, printing inks cosmetic, pharmaceuticals, etc. (Tibor, 2007)**.**

Microbial pigments are a promising alternative source for natural food grade pigments and have a great potential for food application due to their natural color, safe for use and medicinal properties (Francis et al., 2000; Johnson and Schroeder, 1996).

The high production cost of natural colors is likely to change due to production of microbial food grade pigments thus leading to a cheaper source of natural food colorants among the modern consumers. Hence, microbial pigment production is now one of the emerging fields of research and in nature a wide range of color rich and pigment producing microorganisms (bacteria, fungi, yeast, protozoa) (Duffose, 2009) offer scope for commercial production of biopigments (Keneni and Gupta, 2011; Sasidharan et al., 2013; Tarangini and Mishra, 2013; Moss, 2002).[9]

Microbial colors are available in different shades. These colors are biodegradable and environment friendly. They also have numerous clinical characteristics like antioxidant, anticancer, antiproliferative, immunosuppressive, treatment of diabetes mellitus etc.[10]

Actinomycetes play a major role in recycling of organic matter.[11]

Production of novel pharmaceuticals, nutritional materials, cosmetics, enzymes, antitumor agents, enzyme inhibitors, immune-modifiers and vitamins. Streptomyces are especially prolific and can produce a great many antibiotics (around 80% of the total antibiotic production) and active secondary metabolites.[12]

Streptomycetes and related Actinomycetes continue to be useful sources of novel secondary metabolites with a range of biological activities that may ultimately find applications as anti-infectives, anticancer agents or other pharmaceutically useful compounds.[13]

2. MATERIALS AND METHODS

2.1 Sample Collection: Marine water sample collected from Konkan coast was used for the isolation of pigment producing bacteria and Actinomycetes. The samples were collected in sterile glass bottles and brought to laboratory in sterile mineral base media. The samples were stored at -20^{o}C.

2.2 Media: The media used for enrichment and isolation of pigmented bacteria were Zobells marine media, nutrient broth and Mueller-Hinton Agar procured from Hi-Media.

2.3 Isolation and Identification

Collected samples were serially diluted using sterile saline Odd dilutions were spread along with water control, sediment control and media control. Loopful of suspension was streaked on sterile Zobell agar plates and the plates were incubated at 37 °C for 24 hrs. Only the pigmented bacterial colonies and Actinomycetes were selected and sub-cultured. These colonies were observed and colony characters were recorded .The isolates were morphologically identified by Gram staining. Motility was performed and they were further identified using biochemical methods as stated in Bergey's manual for characterization (1994) which includes indole, methyl red, Voges Proskauer, citrate, urease, catalase, oxidase etc. Sugar fermentation test was carried out for glucose and lactose. Slide culture of Actinomycetes was performed on Casein starch agar medium and incubated at room temperature for 72 hrs.

2.4 Screening of Enzymes: The isolates were screened for the production of enzymes such as urease, catalase and oxidase using different plate assays method as detailed below:

Urease Production: The urease test is used to determine the ability of an organism to split urea, through the production of the enzyme urease.

Catalase Production: Catalase is the enzyme that breaks hydrogen peroxide (H_2O_2) into H_2O and O_2.

Oxidase Production This is a test to see if an organism is an aerobe.

2.5 Screening for antimicrobial activity: Antimicrobial activities of isolates were tested preliminarily by cross streak method .Pigmented isolates were streaked across diameter on nutrient agar plates. After incubation for 24 hrs. at 37^{o}C, 24 hrs. old cultures of *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas sp.* were streaked perpendicular to the central strip of pigmented culture. All plates were again incubated at 37^{o}C for 24 hrs. and zone of inhibition was measured.[14]

Extraction of pigment from pigmented bacterial isolates

The organism was grown in sterile nutrient broth in a rotary shaker. Then the cells were harvested and separated by centrifugation. The harvested cells were washed twice and centrifuged. The cell pellet was then suspended in acetone methanol solution (5:5 v/v) and sonicated followed by centrifugation. The colored supernatant was separated then it was filtered through Whatman no. 1 filter paper. The extract was analysed by scanning the absorbance in the wavelength region of 450 nm using the spectrophotometer. The total pigment content in the extract was estimated by measuring the absorbance at λmax (450 nm).[15] DMSO was used as a control.

Biological activity of the crude extract

Antibacterial Assay: To check if the extracted pigments have any antibacterial property, well diffusion method (Bauer et al, 1966) was performed using 2 lab cultures namely *Bacillus subtilis* and *Pseudomonas aeruginosa*.[16]

Mueller Hinton agar (MHA) plates were prepared and previously seeded with the test organism in each plate. The wells were cut by using a sterile cork borer. Forty microliter of

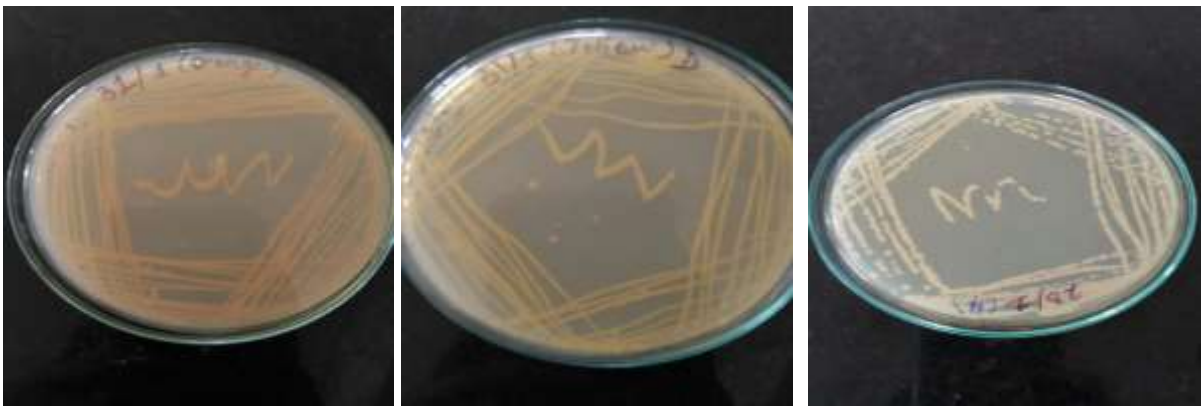
culture filtrate and crude extract of the isolate was added in each well. The diameters of zone of inhibition were determined after 24 hrs of incubation at 37°C for bacteria.[17]

Antibiotic sensitivity

To determine **antibiotic sensitivity**, an active culture was inoculated onto Mueller-Hinton (Hi-media) agar plates, octadiscs impregnated with antibiotics were placed on their surface. The plates were incubated and the zones of inhibition around the antibiotic discs were recorded.[18]

DNA Extraction DNA extraction was performed according to following methods: Cells were cultured to approximately the late exponential phase .The cells were then harvested by centrifugation. The deposit was suspended in solution [glucose 50mM (pH 8.0), Tris-HCl 25 mM (pH 8.0), and EDTA 10 mM]. Cell lysis was done by adding 5% lysozyme and 1% RNase. DNA was extracted by adding extraction buffer (20 mM Tris-HCl, 100 mM EDTA, 1% sodium dodecyl sulfate, 0.01% proteinase K). The lysate was extracted two times with an equal volume of phenol/chloroform/ isoamyl alcohol (25:24:1). Nucleic acid was precipitated with double -volume ethanol and washed with 70% ethanol. Nucleic acid was resuspended in TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8.0).[19]

Isolation and Characterization

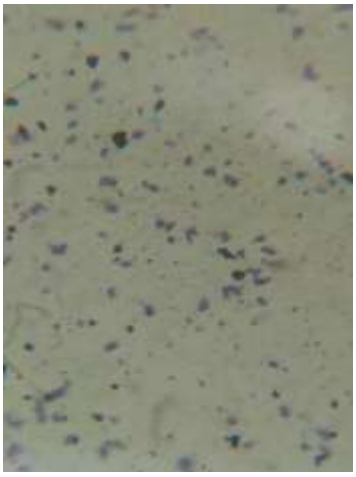


Isolate 1 **Isolate 2** **Isolate 3**

Figure 1: Isolation by Streak Plate Method.

Table 1

Characteristics	**Isolate 1**	**Isolate 2**
Gram staining	Gram positive	Gram positive
Shape	Cocci	Cocci
Motility	Non-motile	Non-motile



Gram Staining of Isolate 1

Gram Staining of Isolate 2

Figure 2: Gram staining of pigmented isolates

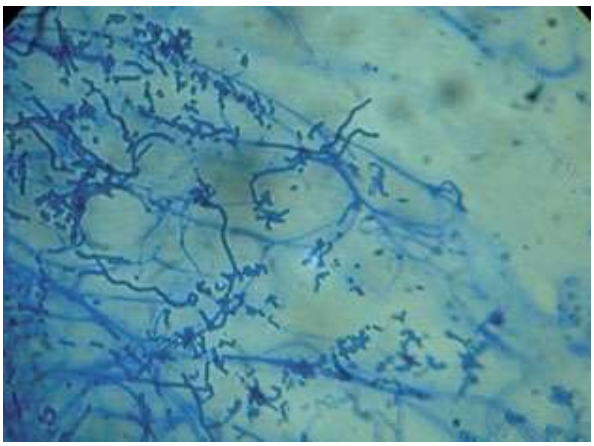
Figure 3: Slide Culture of Isolate 3

Slide culture of Actinomycetes was performed on Casein Starch Agar medium and incubated for 72 hrs. at room temperature.

Microscopic view of slide culture

Objective used: 40 X

Trinocular Microscope, Model AXIO Lab A1.



Gram positive, filaments with hyphae

Table 2: Results of Biochemical Tests

Test	Isolate 1	Isolate 2	Isolate 3
Lactose	-	+	-
Glucose	-	-	+
Urease	+	-	-
Catalase	+	+	-
Oxidase	+	+	+
+ **: Positive** - **: Negative**			

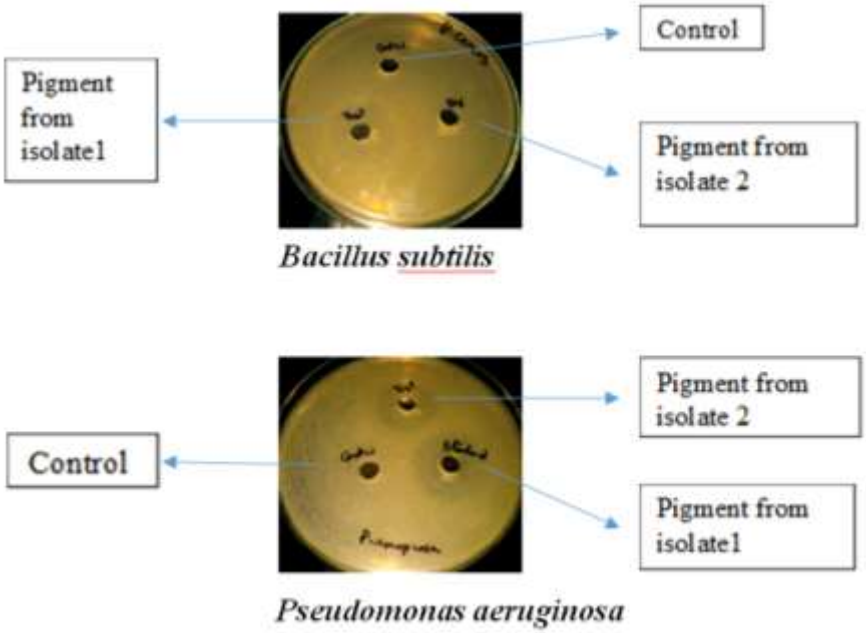


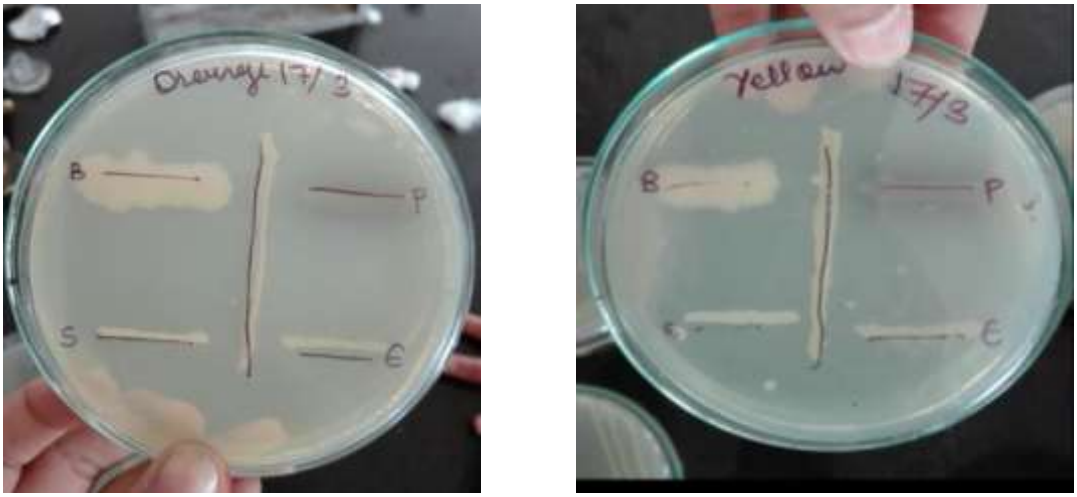
Figure 4: Extraction of orange pigment from Isolate 1

O.D: 0.11 at 450nm

Extraction of yellow pigment from Isolate 2

O.D: 0.65 at 450nm

Figure 5: Antibacterial activity of pigment by Well Diffusion assay method

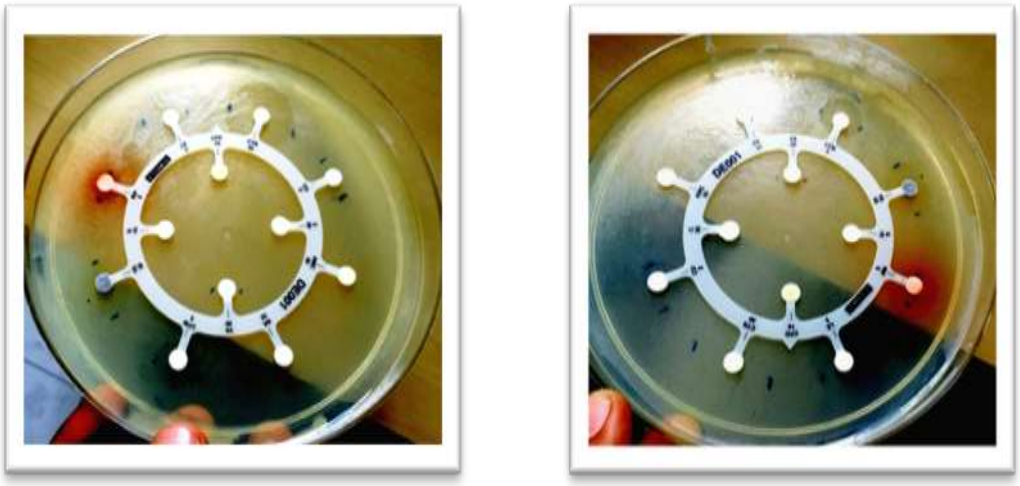


Isolate 1 (Orange) **Isolate 2 (Yellow)**

Figure 6: Antimicrobial activity of pigmented organism by Cross Streak Method

Antimicrobial activity of pigmented isolates was tested against laboratory strains *Bacillus subtilis, Staphylococcus sp., E.coli and Pseudomonas sp.*

Both the isolates were found to be resistant against all 4 organisms.

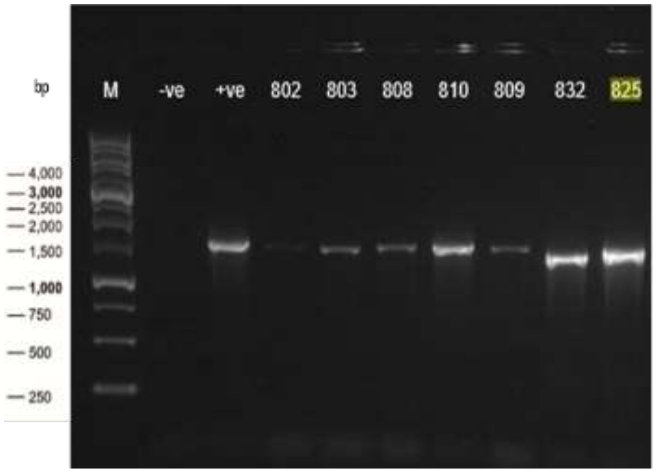


A - Antibiotic disc on lawn of isolate 1 **B - Antibiotic disc on lawn of isolate 2**

Figure 7: Antibiotic Sensitivity Assay- Disc Diffusion Method

Drug Resistance Profile

- Isolate 1 was sensitive against 3 antibiotics, viz, Rifamycin, Ampicillin, and Tetracycline.
- Isolate 2 was sensitive against 6 antibiotics, viz, Rifamycin, Linezolid, Ampicillin, Gentamicin, Ciprofloxacin, Lincomycin.



- **M:** Standard molecular weight marker lane with molecular weights. 4000kb,3000kb, 2500kb, 1500kb, 1000kb, 750kb, 500kb, 250kb
- **+ve Control:** DNA extracted from *E.coli* is used as template
- **-ve Control**: PCR no template control(water to replace template DNA)
- **832** *Streptomyces werraensis* NBRC 13404
- **825** *Salinococcus roseus* DSM 5351
- **802** *Micrococcus endophyticus* YIM 56238

Figure 8: PCR analysis

Isolate 1 was identified as *Salinococcus roseus* using 16S rRNA gene sequencing with 98 to 99 percent similarity. Isolate 2 was identified as *Micrococcus endophyticus* using 16S rRNA gene sequencing with 98 to 99 percent similarity. Isolate 3 was identified as *Actinomycetes*: *Streptomyces werraensis* using 16S rRNA gene sequencing with 98 to 99 percent similarity.

DISCUSSION

Isolation on the abundance distribution of marine bacteria along Konkan coast gives us an idea about diversity among the bacteria with reference to their nature, growth characteristics and pigmentation patterns. Similar work was done by Mukesh et al 2014[20] from India. We obtained three isolates *Salinococcus roseus, Micrococcus endophyticus* and *Actinomycetes*: *Streptomyces werraensis.*

Micrococcus endophyticus showed yellowish colonies. The bacterium *Micrococcus endophyticus* was identified as Gram-positive coccus which resembles to the work done by S. Benjamin *et al* 2016.

Pigment from Micrococcus endophyticus and Salinococcus roseus showed antimicrobial activity which was also reported by Azamjon B et al 2011.[2]

Isolated organisms showed similar antibiotic sensitivity as mentioned in the work done by Chen et al 2009.[21]

However, it is widely accepted that current and traditional culture based techniques are not adequate to study microbial diversity from environmental samples. Our understanding of marine microbial communities has increased over the past two decades as result of culture independent phylogenetic studies (Fox et al., 1977). Recent advances in molecular techniques

are sufficient to describe the microbial diversity in a marine sample based on 16S rRNA sequence diversity. It has been proved that the exploitation of molecular biological techniques will help in understanding and addressing different research questions about marine organisms and ocean processes. Marine bacteria belonging to the order Actinomycetales have been proved as producer of a wide range of natural products.[1]

Hence molecular biology methods are the only reliable tools but the limitation is that they are time-consuming and not cost-effective.

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