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Studies on bioactive prodigiosin of serratia marcescens isolated from natural sources

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Abstract

Prodigiosin is a natural red colored pigment that specifically found to produced by many strains of *Serratia marcescens* and other related organisms as a secondary metabolites. Prodigiosin has a wide variety of biological activities and hence its efficacy optima strive researcher. Our present study aimed to determine antimicrobial, antifungal and antibiofouling activities of prodigiosin produced by *Serratia marcescens* isolated from various natural sources. Among the red pigment producing isolates, S_8isolate, a higher pigment producer, was selected for further studies (was identified as *Serratia marcescens* by 16S rRNA sequencing, NCBI Accession No. MH145360). From 16S rRNA analysis, phylogenetic tree was constructed to study its phylogenic relationship. Antimicrobial activity of pigment showed reveals its effectiveness against gram positive organisms. Prodigiosin showed antifungal activity against *Aspergillus niger, Mucor hiemalis, Fusarium enumartii* and *Rhizopus stolonifer*. Prodigiosin was also known to reduce the adhesion of biofouling organisms such as *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*, hence can be used as antibiofoulant.

Keywords: Prodigiosin, *Serratia marcescens*, Antimicrobial activity, Microbial pigments, Biopigment, Red pigment

Introduction

A variety of secondary metabolites are produced by many organisms. These secondary metabolites has important applications as enzymes, pigments, steroids, antibiotics, etc and are produced in industries on large scale. Color is any dye, pigment or other substance that can impart color to food, drug or cosmetic or to the human body (Kirti et al., 2014). Color affects every single thing of life which includes not only the food we eat but the things we use in our day to day life (Downham and Collins, 2000). Let us think for a minute that how plants can make their own food without chlorophyll and how oxygen could be carried in the body without haemoglobin. So it can be said that life on earth is totally dependent on pigments (Brilton, 1995).

Natural pigments as well as synthetic dyes are used in many different fields such as food, textile, printing, paper, inks, cosmetic,

pharmaceutical, etc (Goswami and Bhowal, 2014). There are three main sources of natural pigments: Plants, Animals and Microorganisms. Microbial pigments are the natural pigments preferred the most among other natural sources of pigments. This is due to several advantages offered by them: Fast growth rate, Easy doubling time, Cheap culture medium, Stability of microorganisms, Availability of cultural conditions throughout the year, Different shades of color are obtained, High yield and Lower residues, etc (Hariyali and Manisha, 2018).

Prodigiosin from *Serratia marcescens* is one such biopigment among several natural biopigments produced by different microorganisms. Prodigiosin is a red colored pigment which has a tripyrrole in its structure and belongs to the family Prodiginines. *Serratia marcescens* is a gram negative, facultative anaerobic, motile, single short rods which belongs to the family Enterobacteriaceae. As Prodigiosin has showed many different biological activities they have got an important place in the fields of medicine, pharmaceutics, textile and different industries.

The present study therefore was conducted to evaluate the antimicrobial, antifungal, and antibiofouling activity of prodigiosin from *Serratia marcescens.* A method of preventing the accumulation of fouling organisms is defined as Antibiofouling.

Materials and methods

A. Isolation and Identification of *S. marcescens*

Samples were collected from various natural sources (Soil, Water, Effluent water, Sewage sludge, and Surface swab). Isolation of red pigment producing bacteria was carried out using spread plate technique. Pure cultures of isolates were obtained by sub culturing and were preserved on Nutrient agar slants at 4^0C. Potential isolate among all isolates was selected for further study. The selected isolate was identified by 16S rRNA gene sequence analysis carried out commercially from Saffron lifescience, Bilimora, Navsari (Gujarat). The partial sequence of 16S rRNA of selected isolate was deposited in NCBI database. Phylogenetic analysis was carried out.

B. Production, Extraction and Confirmation of the pigment

(1) Inoculum preparation

Inoculum preparation was carried out in 50 ml nutrient broth. Nutrient broth was inoculated with a single colony of the selected isolate and incubated at 30^0C on a rotary shaker for 24 hours.

(2) Production of the pigment

100 ml nutrient broth was prepared in 250 ml Erlenmeyer flask and was then inoculated with the inoculum (2% v/v) and incubated at 30^0C on a rotary shaker at 120 rpm for 24-48 hours.

(3) Extraction of the pigment

The pigment produced by the isolate was insoluble and intracellular and therefore pellet rather than the supernatant was used for extraction of pigment. The bacterial cell absorbance in the broth was measured at 620nm (Gulani et al, 2012). The culture broth was centrifuged at 9000 rpm for 10 minutes at 4^0C and the cell pellet was collected. Extraction of the pigment was done using acidified methanol (1N HCl 1 ml: Methanol 24 ml) (Rakh et al, 2017). Acidified methanol was added to the cell pellet, vortexed and centrifuged at 9000 rpm for 10 minutes at 4^0C. The supernatant was collected in a sterile test tube.

(4) Confirmation of the pigment

The supernatant was used for confirmation of the pigment. 2 ml of the supernatant was taken in two different tubes. Content of one

tube was acidified using a drop of concentrated HCl while the content in the other tube was alkalinized using a drop of concentrated ammonia. This test is called as the Presumptive test for the confirmation of the pigment as prodigiosin (Gulani et al, 2012).

C. Characterization of the pigment

Characterization of the pigment was done by determining the absorbance maxima of the pigment, estimating the pigment (U/Cell) as well as FT-IR analysis.

(1) Determination of Absorbance maxima of the pigment

Spectral analysis of pigment was carried out by UV-Vis Spectrophotometer in the range of 300-700nm using acidified methanol as blank and ʎmax was determined (Geetha et al, 2014).

(2) Estimation of the pigment

Bacterial cell absorbance and pigment absorbance was noted at every step (Kamble and Hiwarale, 2012). The pigment was estimated using the following formula (Mekhael and Yousif, 2009):

$$Prodigiosinunit/cell = \frac{[(OD534 - (1.381 \times OD620)] \times 1000}{OD620}$$

Where,
OD534 – pigment absorbance
OD620 – bacterial cell absorbance
1.381 – constant

(3) Fourier- Transform Infrared Spectroscopy (FT-IR Spectroscopy)

Fourier- Transform Infrared Spectroscopy, also simply called as FT-IR Spectroscopy, is a technique that provides information about the chemical bonding or molecular structures of materials, whether organic or inorganic. The technique works on the fact that bonds and group of bonds vibrate at characteristic frequencies. A molecule that is exposed to infrared rays absorbs infrared energy at frequencies, which are characteristic to that molecule. During FT-IR analysis, a spot on the specimen is subjected to a modulated IR beam. The specimen's transmittance and reflectance of the infrared rays at different frequencies is translated into an IR absorption plot consisting of reverse peaks. The resulting FT-IR spectral pattern is then analysed and matched with known signatures of identical materials.

D. Evaluation of Bioactivities

(1) Antimicrobial activity of prodigiosin

The antimicrobialactivityof the extracted pigmentwasevaluated using well diffusion assayonNutrient agar (Valgus et al, 2007). 20 ml of the melted nutrient agar was taken and cooled to room temperature. Then 0.1 ml of the test organism was inoculated in it, mixed well and poured into sterile Petri dish. After the agar solidifies, 8mm well were bored in the agar. 100 µL of the extract was then filled in the well. Methanol was taken as negative control and Chloramphenicol (30mg/ml) was taken as standard. The plates were kept at room temperature for diffusion of extract before the growth of the organism (Pre-diffusion time). The plates were then incubated at 37^0C for 24 hours and zone of inhibition was measured in millimetres. The test organisms used included Gram positivebacteria *Staphylococcusaureus*(MTCC No. 737),*Bacillus subtilis* (MTCC No. 441) and*Bacillusmegaterium*(MTCC No. 2949) and Gram negative bacteria *Escherichia coli*(MTCC No. 118.119), *Pseudomonas aeruginosa*(MTCC No. 2297) and*Salmonella paratyphi A*(MTCC No. 735).

McFarland turbidity standards were followed for comparing the density of the bacterial suspensions.

Table no-1: McFarland turbidity standards

McFarland turbidity standard no.	0 . 5
1% Barium chloride (ml)	0 . 0 5
1% Sulphuric acid (ml)	9 . 9 5
Approx. Cell density ($1\times18^{\Lambda}$ CFU/ml)	1 . 5

0.5 McFarland turbidity standard was used for antimicrobial test. Density of the bacterial suspension was compared with 0.5 McFarland turbidity standard by holding both the tubes in front of light against a white background having contrasting black lines. In case of too high turbidity, the suspension is diluted using sterile distilled water, saline or both. In case of low turbidity, bacterial cells are added to it. The suspensions should be used within 15 minutes.

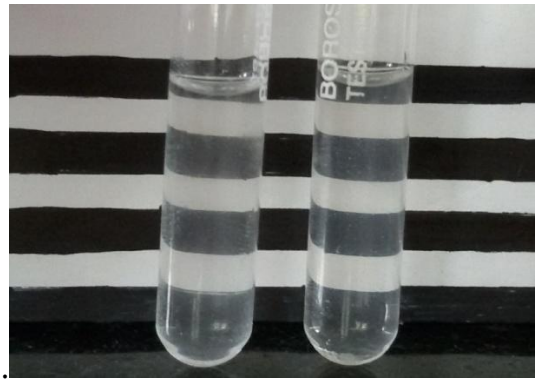

Figure-1: Matching turbidity standards

(2) Antifungal activity of prodigiosin

Antifungal activity of prodigiosin was checked against different fungal pathogens by agar well diffusion method. The fungi used included *Aspergillus niger* (MTCC No. 281), *Mucor hiemalis* (MTCC No. 157), *Fusarium enumartii* (MTCC No. 399) *and Rhizopus stolonifer* (MTCC No.162). The cultures were spread on Potato dextrose agar plates and 8mm well were bored. 100 µL of the extract was filled in the well. The plates were kept at room temperature for diffusion of extract before the growth of the organism (Pre-diffusion time). The plates were then incubated at 30^0C for 4-5 days. Methanol was used as control (Pankaj et al, 2015). After incubation the zones of inhibition were measured in millimeters.

(3)Antibiofouling activity of prodigiosin

Antifouling is defined as a method of preventing the accumulation of fouling organisms (Sathish and Aparna, 2014). Wood pieces were used as substrate for biofilm formation. The methanol extract of prodigiosin was applied over the wood pieces and allowed to dry. Wood pieces were then immersed in 100 ml marine water which was inoculated with 1 ml of fouling organisms such as *Escherichia coli*(MTCC No. 118.119),*Staphylococcus aureus*(MTCC No. 737),*Pseudomonas aeruginosa*(MTCC No. 2297). After 7 days period, the biofilm was scraped from the surface of wood and SPC was done. Wood piece coated with methanolwas used as a control. The number of the colonies were counted and compared with control and results were noted (Sathish and Aparna, 2014).

Results and discussions

Prodigiosin, a secondary metabolite, has no clear defined function in the organism that produces it, that is in *S. marcescens*. However prodigiosin is known to have wide applications in textile, food cosmetic and therapeutic industries. It is also known to have a variety of biological activities such as antibacterial, antifungal, antioxidant, antibiofouling, anticancer, antimalarial, etc.

A. Isolation and Identification of *S. marcescens*

Total 22 samples were collected from different location (Soil, Water, Effluent water, Sewage sludge, Surface swab, etc). Total 6 isolates (S_8, S_{14}, S_{15}, S_{17}, S_{18}, S_{19})

were isolated from 22 samples. Out of 6 isolates, Isolate S_8 (isolated from effluent water) was found to give maximum amount of red pigment production on agar medium. Therefore it was selected for studying different aspects.

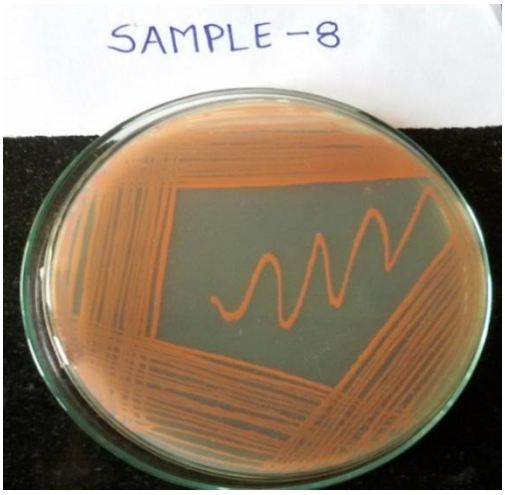

Figure-2: Isolate S_8 on Nutrient agar

Identification of isolate $S_8$was carried out by comparing the partial 16S rRNA sequence with known sequences contained within large database using BLAST tool of NCBI. Partial sequence of the isolate showed 100% identity with 16S rRNA partial sequence of *Serratia marcescens* strain NBRC 102204 (NCBI accession No.NR_114043.1) and identified as *Serratia marcescens*. The partial sequence of 16S rRNA of isolate S_8 was deposited in NCBI database with their accession number MH145360.

The phylogenetic tree was constructed for isolate S_8 by neighbour- joining method. The reliability of phylogenetic tree was assessed by boot strap analysis of 500 replicons.

B. Production, Extraction and Confirmation of the pigment

Production of the pigment by Isolate S_8 was carried out by submerged fermentation in Nutrient broth as it supported maximum pigment production. Inoculum was first developed in Nutrient broth in jumbo tube (15 ml) which were then used to inoculate (1 ml) production medium. Production of pigment was carried out in 250 ml Erlenmeyer flask containing 100 ml nutrient broth.

Extraction of the pigment can be carried out by using various solvents such as methanol ethanol, acetone, chloroform etc. But as per our review methanol as well as acidified methanolgives best results and so we used it as a solvent in our study. In present study acidified methanol as solvent proved best while Rakh et al. in 2017 used 95% methanol for the extraction of pigmentand Venil et al., in 2017 used ethyl acetate and acetone for extraction of pigment. Similar to our study Krithika and Ramani in 2013 also used 95% methanol for pigment extraction.

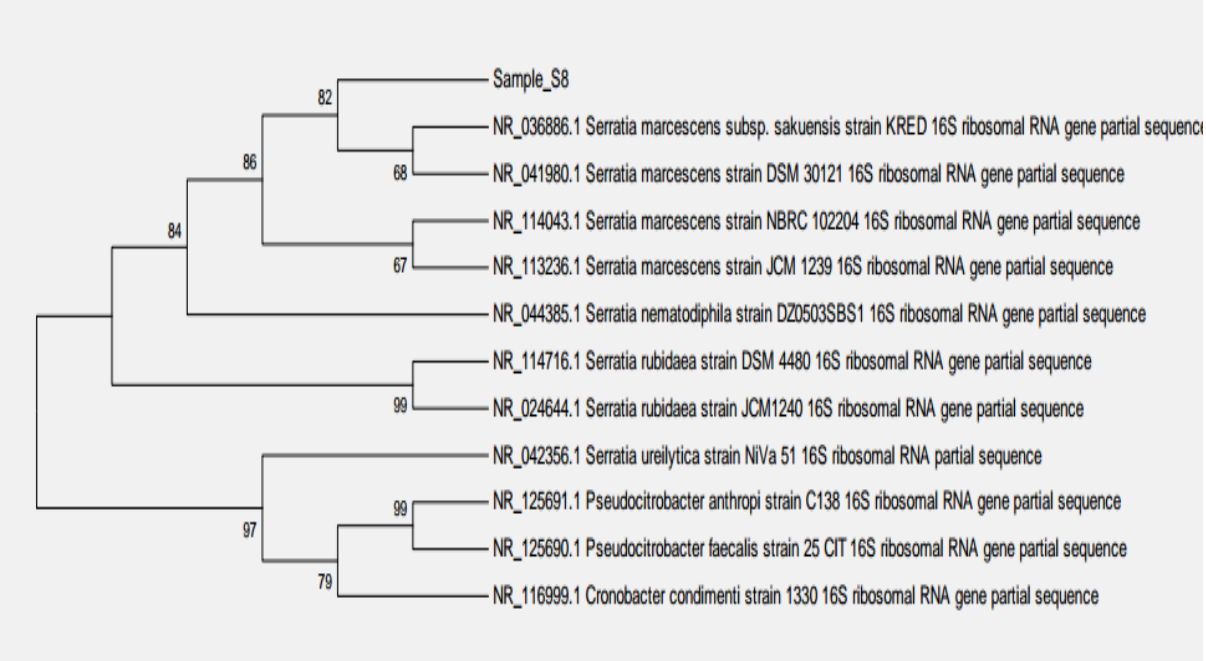

Figure-3: Phylogenetic tree showing position of isolate S_8 with related organisms based on 16S rRNA sequences. The optimal tree with the sum of branch length= 0.07640080 is shown. The confidence probability (multiplied by 100) that the interior branch is greater than 0, was estimated using the bootstrap test (500 replicates) and are shown next to the branches. The evolutionary distances were computed using the maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 959 positions in the final dataset.

Figure-4: Extracted pigment using acidified methanol as solvent

Presumptive test was performed for the confirmation of pigment which was done by acidified and alkalinized condition. A red/pink color in acidic condition and yellow/tan color in alkalinized condition was observed after addition of one drop of concentrated HCl and concentrated ammonia in two different tubes containing pigment respectively which indicates a positive presumptive test for pigment and confirms pigment as prodigiosin.

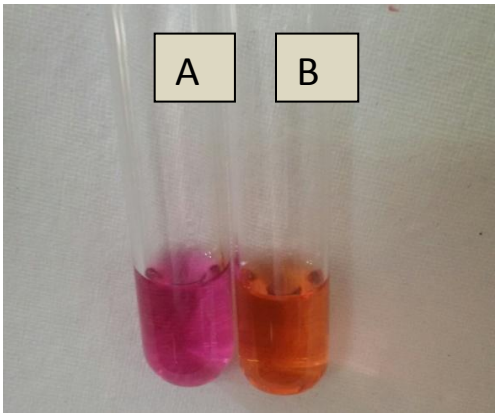

Figure-5: Positive presumptive test: [A]- Acidic condition [B]- Alkalinized condition

C. Characterization of the pigment

Characterization of the pigment was done by determining absorbance maxima, its estimation (U/cell) and FT-IR analysis.

(1) Determination of Absorbance maxima of the pigment

The pigment after extraction was characterized by studying the absorption spectra of the pigment. The pigment was subjected to UV-Vis spectrum analysis in the range between 300-700 nm using acidified methanol as blank. Maximum absorbance peak of the pigment was observed at 534 nm. Similar studies were carried out by Gulani et al., in 2012 where the methanolic extract of prodigiosin showed characteristic maxima at 499 nm. Rakh et al., in2017 observed that the pigment extracted using 95% ethanol when acidified with 1N HCl showed a single peak absorbance at 535 nm and when alkalinized with 1N NaOH showed single peak absorbance at 470 nm.

(2) Estimation of the pigment

Cell density of the broth was measured by us after pigment production. Pigment was estimated according to the formula given byMekhael and Yousif in 2009. The prodigiosin estimated in Nutrient broth was found to be 6528.81 Prodigiosin unit/ cell but in contrast Gulani et al in 2012 found peptone glycerol broth as best medium for prodigiosin production and prodigiosin estimated was 763.55 prodigiosin unit/cell.

(3) Fourier- Transform Infrared Spectroscopy (FT-IR Spectroscopy)

From the spectrum obtained, red pigmented prodigiosin showed a broad envelope around 3600- 3300 cm^{-1}centered at 3302 cm^{-1} attributed to the -N- H stretch. The peaks at 2903 and 2864 $cm^{-1}$are due to asymmetrical and symmetrical stretching of methylene groups. The peaks at 1652 and 1456 $cm^{-1}$are due to the presence of –NH and methyl groups. The visible peak at6 1396 cm^{-1} is due to the presence of C-O group in prodigiosin. The peaks around 1232 and 705 cm^{-1} are attributed to carbon carbon double bond.

D. Evaluation of Bioactivities

(1) Antimicrobial activity of prodigiosin

The antimicrobial potency of the prodigiosin was tested against Gram positive and Gram negative organisms using well diffusion assay. It was observed that prodigiosin was able to inhibit majority of the test organisms. Prodigiosin possess a good antimicrobial activity against gram positive organisms rather than gram negative organisms. The highest zone if inhibition was found against *Staphylococcus aureus*while the lowest against *Escherichia coli*. Zone of inhibition against test organisms are given in the table below.

Table-2: Results of Antimicrobial activity of prodigiosin

Test organism	Diameter of Zone of inhibition (mm)					
	Positive control (Chloramphenicol-30 mg/ml)		Negative control (Methanol)		Red pigment Prodigiosin (in methanol)	
Gram positive organism						
Bacillus subtilis(MTCC No.441)	2	5	0	0	1	5
Bacillus megaterium(MTCC No. 2949)	1	8	0	0	1	3
Staphylococcus aureus(MTCC No. 737)	1	5	0	0	1	6
Gram negative organism						
Escherichia coli(MTCC No. 118.119)	1	6	0	0	1	1
Pseudomonas aeruginosa(MTCC No. 2297)	2	2	0	0	1	3
Salmonella paratyphi A(MTCC No. 735)	1	8	0	0	1	5

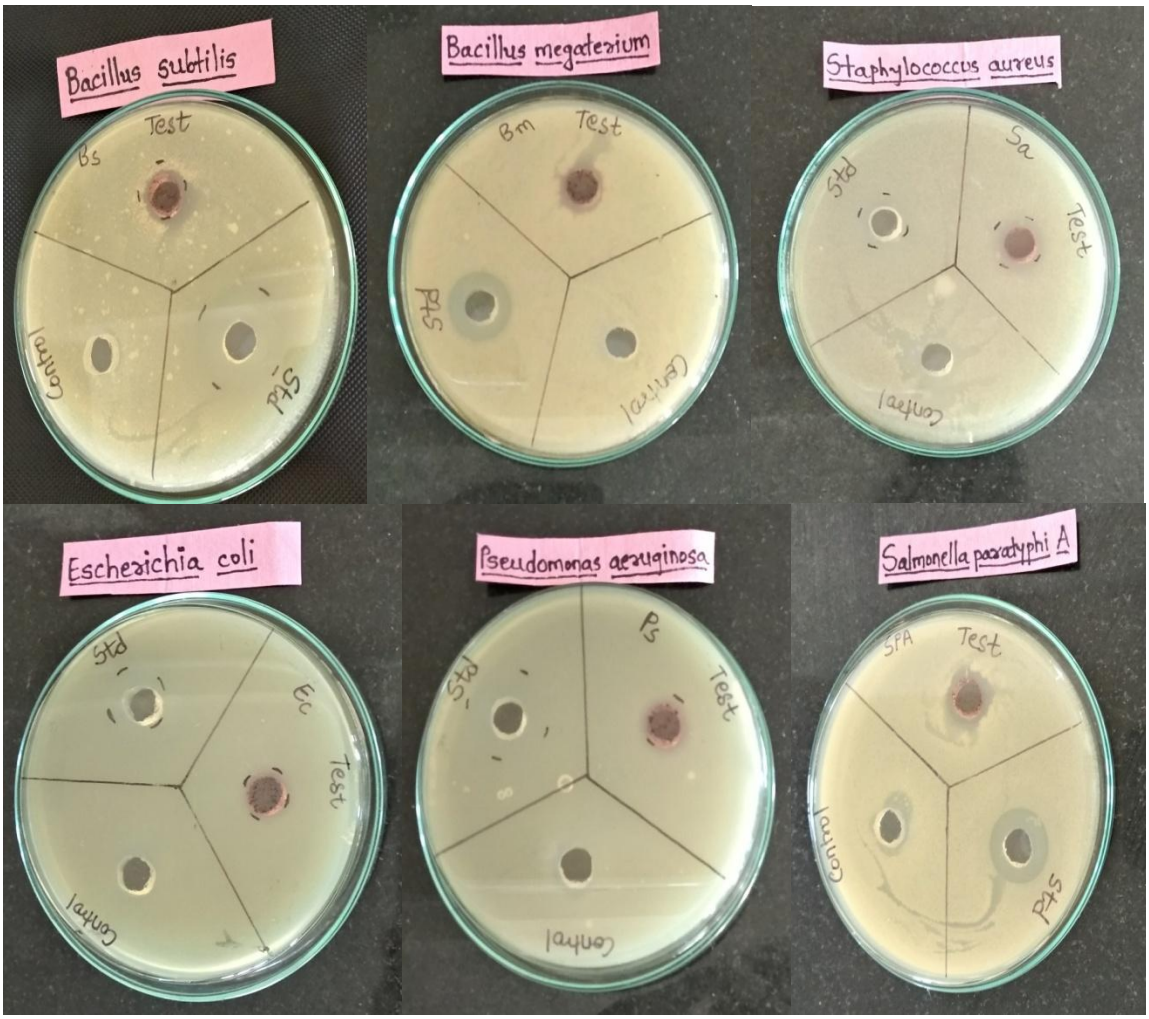

Figure-6: Antimicrobial activity of prodigiosin against gram positive and gram negative organisms.

(2) Antifungal activity of prodigiosin

Antifungal activity of prodigiosin was checked against *Aspergillus niger* (MTCC No. 281), *Mucor hiemalis* (MTCC No.157), *Fusarium enumartii* (MTCC No.399) and *Rhizopus stolonifer* (MTCC No.162). Clear zone of inhibition was observed. The results of antifungal activity are given below in the table.

Table-3: Results of Antifungal activity of prodigiosin

Test organism	Diameter of Zone of inhibition (mm)
Aspergillus niger (MTCC No. 281)	1 7
Mucor hiemalis (MTCC No. 157)	2 0
Fusarium enumartii (MTCC No. 399)	2 4
Rhizopus stolonifer (MTCC No. 162)	2 1

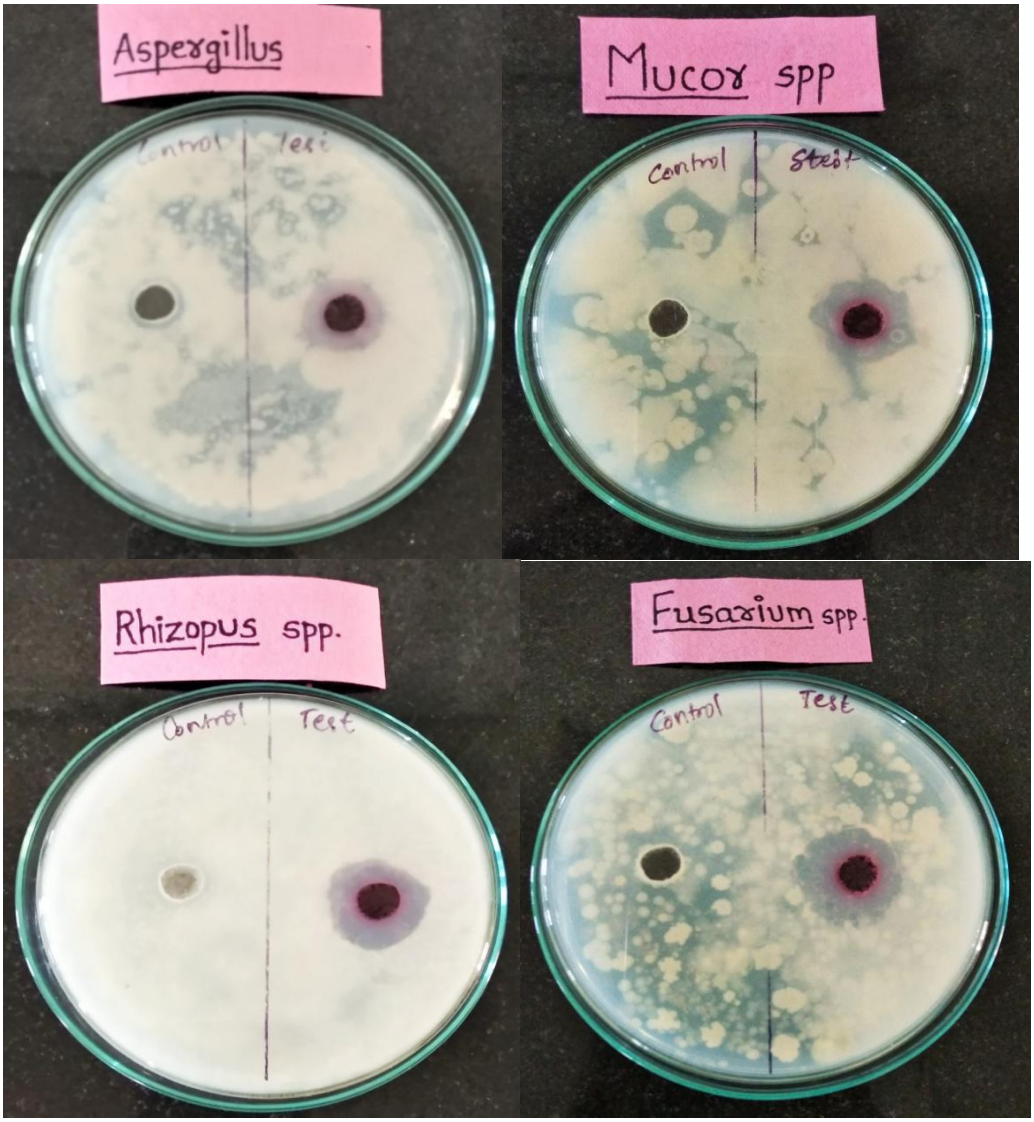

Figure-7: Antifungal activity of prodigiosin

(3) Antibiofouling activity of prodigiosin

Anti-biofouling activity of the prodigiosin was checked. At the end of 7th day, the substrate (wood pieces) were removed from the marine water and enumeration of bacteria was carried out. No. of the colonies obtained from the pigment coated substrate were counted and compared with the no. of the colonies obtained from methanol coated substrates. It was found that there was difference in the number of the colonies. The experimental setup for biofilm formation is shown in the figure.

Figure-8: Experimental setup for biofilm formation

The pigment coated wood was found to significantly reduce the adhesion of thebiofouling organisms. This showed that application of pigment has reduced the number of biofilm forming bacteria. So the pigment can be efficiently used as Antibiofoulant.

Table-4: Comparison of the number of the colonies on pigment coated and methanol coated wood

Organism	No. of the colonies				
	Methanol coated wood			Pigment coated wood	
S. aureus(MTCC No. 737)	1	5	4	9	4
E. coli(MTCC No. 118.119)	1	8	3	7	9
P. aeruginosa(MTCC No. 2297)	1	3	5	7	5

Conclusion

The bacterium isolated from effluent sample was identified by 16S rRNA gene sequence analysis as *Serratia marcescens*, a member of Enterobacteriaceae family. *S. marcescens* produced a biopigment "Prodigiosin" which has potential antimicrobial, antifungal and antibiofouling activity.

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