International Journal of Biology Research
www.biologyjournal.in
ISSN: 2455-6548
Received: 03-09-2022, Accepted: 18-09-2022, Published: 07-10-2022
Volume 7, Issue 4, 2022, Page No. 1-4

Isolation of *Serratia marcescens*, its pigment extraction & study of antimicrobial activity of extracted pigment

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Abstract
Pigments are natural as well as manmade biochemical compounds produced by plants and microorganisms which exhibits specific colors. [1] They are mostly used in dye and textile industries, cosmetics and also in medicines due to its antimicrobial abilities. [1] *Serratia marcescens* belongs to a group of pigmented bacteria which produces a pigment called as "Prodigiosin" – a secondary metabolite. In this study, the bacteria was isolated from various samples (soil, water, coconut) on nutrient agar supplemented with glycerol which act as growth enhancers for the bacteria. Strains which shows red pigmentation were selected. Various morphological and biochemical tests were performed for identification of the strain. Extraction of the pigment was carried out using plate-swabbing methanol method, where methanol was used as a solvent. The extracted pigments were diluted in distilled water and the antimicrobial effect of the pigment was studied against *Escherichia coli, Staphylococcus aureus and Salmonella typhi* using agar-cup method. The clear zone of inhibition that formed around the well indicates antimicrobial activity of the pigment.

Keywords: serratia marcescens, isolation, pigment, prodigiosin, plate-swab method, antimicrobial activity, agar-cup method

Introduction
Natural pigments are sourced from ores, insects, plants and microbes. Biopigments produced from microorganisms are preferred over those from plants because of their stability and availability for cultivation throughout the year. Among microbes, bacteria have immense potential to produce diverse bioproducts and one such bioproduct is pigments [2]. These bacteria can be isolated/ cultured/purified from various environmental sources such as water bodies, soil, on plant, in insects and in man or animal. Various growth mediums can be used to isolate different types of bacteria.
Serratia marcescens is a Gram negative, bacillus shaped bacteria that belongs to the family *Enterobacteriacea*. These bacteria grow well on standard media and produce a red to dark pigment that aids in its identification and the red color pigment is so called 'Prodigiosin' [1].
Prodigiosin is an alkaloid secondary metabolite with a unique tripyrrole chemical structure. It is isolated from few species such as *Serratia, Pseudomonas* and *Streptomyces.* It is sensitive to light and soluble in water & methanol.
The pigment has been reported to have antifungal, antibacterial, algicidal, antiprotozoal, antimalarial activity, immunosuppressive and anticancer activities [1].
The production of Prodigiosin in *Serratia marcescens* strains is susceptible to temperature and is substantially inhibited at temperatures higher than 37°C. Conventional media used for the biosynthesis of Prodigiosin by *Serratia marcescens* strains are nutrient agar supplemented with glycerol.

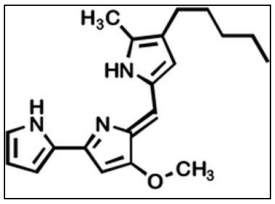

Fig 1: Prodigiosin

The Prodigiosin group of natural products is a family of tripyrrole red pigments that contains a common 4-methoxy, 2,2- bipyrrole ring system. The biosynthesis of the pigment is a bifurcated process in which mono and bipyrrole precursors are synthesized separately and then assembled to form Prodigiosin.
Prodigiosin have been shown to be associated in extracellular vesicles, cell associated or present in intracellular granules [3].

Prodigiosin production conditions (media, temperature and carbon source)
Nutrient broth, peptone glycerol broth, regular growth media which facilitates Prodigiosin production [3].
The maximum yield of Prodigiosin is seen in crushed sesame seed broth with incubation at 28°C, 30°C whereas, in nutrient broth and peptone glycerol broth, the maximum yield was seen at 28°C and 30°C. At 37°C Serratia marcescens does not show any pigment production [3].

Materials and Methods
Isolation of *Serratia marcescens* from various sources
The samples selected for isolation of pigmented bacteria were soil sample, water sample and white part of wet coconut. For isolation from soil sample, a serial dilution using 0.9% sterile saline, followed by spread plate method was performed. For isolation from coconut, the samples were directly streaked onto nutrient agar or were inoculated into nutrient broth.
The plates were incubated for 24 to 48 hrs at 28ºC to obtain pigmented colonies of *Serratia marcescens.*

Identification of the bacteria
For identification of bacteria, morphological and biochemical tests were performed. In morphological test

colony characteristic on nutrient agar were observed and microscopic observations were done through gram staining.
In biochemical tests, Indole test, Methyl-red test, Vogues Proskauer test, Citrate test [IMViC] and Catalase test were performed.

Enrichment for Prodigiosin production
Loopful of the colonies from previous nutrient agar plates were inoculated in sterile peptone water for half an hour and then swabbed onto a fresh nutrient agar plate supplemented with 2% glycerol and 2% mannitol which provide enrichment to get maximum yield of the pigment, the plates were incubated at RT for 48hrs.

Extraction of the pigment
The 48hrs old bacterial cells grown on Enriched nutrient agar were scrapped using scalpel and were hydrolyzed in 30-40ml methanol and kept overnight in refrigerator. After 24hrs, the cells were centrifuged at 2500 rpm for 15mins. The supernatant was collected as the extracted pigment.

Determination of antimicrobial activity of the pigment
The method used for this was agar cup method/ agar well diffusion method. Bacterial cultures such as *E. coli*, *S. aureus* and *Salmonella typhi* were selected for testing their susceptibility towards the extracted pigment. These bacterial cultures were suspended in sterile saline to obtain O.D. of 0.5 at 545nm.
Mueller Hinton agar plates were prepared and 0.1ml of each of the above cultures were bulk seeded into 20ml of sterilized molten and cooled MH agar butt. The agar was poured in sterile petri plates and solidified. After solidification, wells were punched by sterile metal cork borer of internal diameter 10mm.
The following dilutions of the pigments [1:1, 1:5 and 1:10] were prepared using D/W as the diluent and added to different wells of the plates. Control wells were maintained on the plate with D/W being the negative control and standard antibiotic as the positive control. All the plates were incubated at 37^{o}C for 24hrs.
After 24hrs the antimicrobial activity was evaluated by measuring the diameter of zone of inhibition which was expressed in millimeter (mm).

Results and Discussion
Isolation of pigmented bacteria from various sources
The soil samples were diluted in sterile saline and streaked onto nutrient agar. After 24hrs of incubation red coloured colonies were selected for further procedures. [Fig 1]
For bacterial isolation white meat part of the coconut were exposed to air and directly streaked onto nutrient broth and red colored colonies were selected for further procedures.

Identification of the bacteria
The isolates were microscopically tested by gram staining and pink coloured rod were observed which conforms that the isolated bacteria is a gram negative bacteria.
The biochemical test performed Indole and methyl-red test as negative and VP, Citrate and Catalase test as positive [Fig 2]

Enrichment and Extraction of pigment
The organism were enriched on nutrient agar plate by swabbing the culture mixed in peptone water which gives higher nutrient value to the medium and also enhances the pigment production. The addition of mannitol to the medium acts as extra sugar source whereas; addition of glycerol enhances the pigment production [Fig.3].
The extraction process involves methanol or ethanol [Fig.4] as they are sterilizers and brings out cell lysis and death which leads to pigment extraction from the organism. The methanolic extract when centrifuged leads to settling of the cell debris to the pellet and the supernatant contains pure extracted pigment. This supernatant i.e. the extracted pigment [Fig.5] can be stored in a beaker covered with foil paper and can be preserved for further use in refrigerator.

Determination of antimicrobial activity of the pigment
The antimicrobial activity of the pigment was determined using agar cup method. The pigments were added in different dilutions using d/w as diluent. [Fig 6] d/w and Std. Antibiotic were used as negative and positive control respectively.
According to the literature, the pigment is said to have the antimicrobial activity against both gram positive and gram negative organism. The bacterial cultures used for the assay were E. coli, S. aureus and Salmonella typhi. Table 1 shows the results for the antimicrobial activity of the extracted pigment. Fig 7a, 7b and 7c shows the zone of inhibition of the pigment *against E. coli*, *S. aureus* and *Salmonella typhi* respectively.

Fig 2: Isolated bacteria from soil and coconut

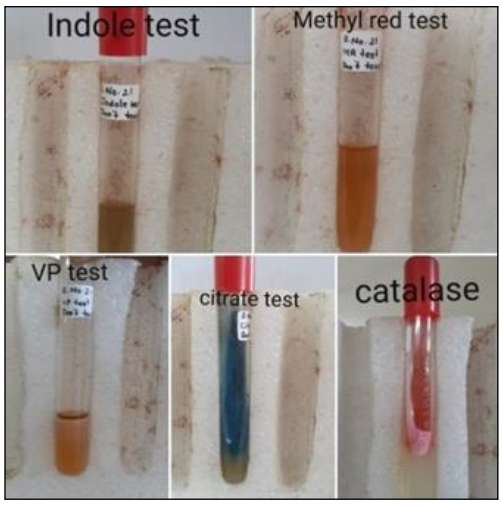

Fig 3: Biochemical Tests

Fig 4: Enrichment of the pigment on nutrient agar supplemented with glycerol &mannitol

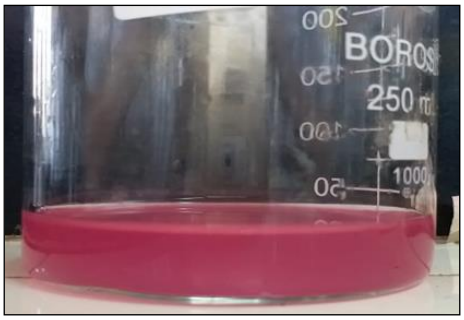

Fig 5: Supernatant: Extracted pigment

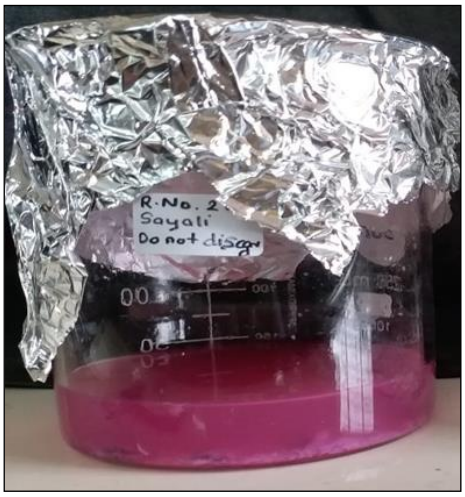

Fig 6: Enriched colonies scrapped into methanol for pigment extraction

Fig 7: Solubility of the pigment in distilled water

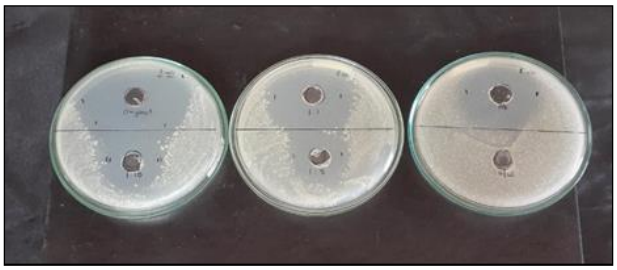

Fig 8a: Antimicrobial activity of pigment against *E. coli*

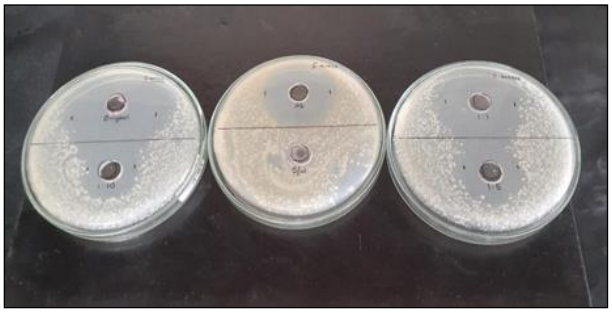

Fig 8b: Antimicrobial activity of pigment against S. aureus

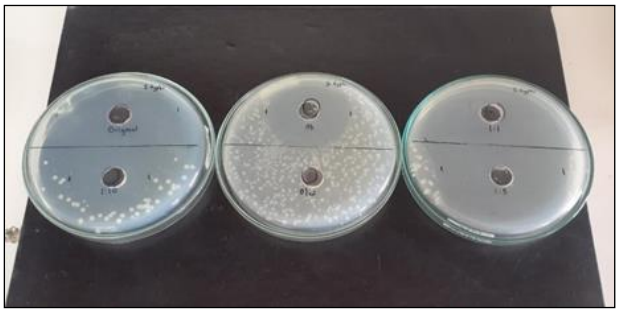

Fig 8c: Antimicrobial activity of pigment against *S. typhi*

Table 1: Antimicrobial activity of the pigment

Name of the organism	Volume of pigment	Zone of inhibition (mm)
E. coli	Control	10mm
	Original	56mm
	1:1	38mm
	1:5	27mm
	1:10	25mm
	Std. antibiotic solution	38mm

Table 2

Name of the organism	Volume of pigment	Zone of inhibition (mm)
S. aureus	Control	10mm
	Original	47mm
	1:1	37mm
	1:5	27mm
	1:10	25mm
	Std. antibiotic solution	37mm

Table 3

Name of the organism	Volume of pigment	Zone of inhibition (mm)
S. typhi	Control	10mm
	Original	64mm
	1:1	80mm
	1:5	62mm
	1:10	40mm
	Std. antibiotic solution	46mm

Conclusion

Serratia marcescens was isolated on nutrient agar from various sources such as soil and white meat part of the coconut. The Gram staining performed showed pink colored rods which indicate the organism to be Gram negative in nature. The organism showed positive Vogues-Proskauer test, Citrate test and Catalase test, whereas negative Indole test and methyl red test, indicating its Gram negative nature. Thus, from biochemical tests and Gram staining, it can be concluded that *Serratia marcescens* is a Gram negative organism. The pigment was extracted using plate-swabbing method.

The antimicrobial activity of the pigment was determined by agar cup method. Accurate or appropriate results were obtained when distilled water is being used as the diluent instead of methanol.

From the results obtained, it can thus be concluded that when compared with standard antibiotic solution i.e. Ciprofloxacin, the pigment showed same zone of inhibition for 1:1 dilution for both *E. coli* and *S. aureus*. This means that Prodigiosin can be used as an alternative for ciprofloxacin at lesser dilutions against these two organisms.

Larger zones of inhibition were obtained for all the dilutions when tested against *S. typhi.* Thus, it can be concluded that the maximum antimicrobial activity of the pigment is against *S. typhi* i.e. the Prodigiosin pigment can be used as an alternative to chemical antibacterial agents for treating typhoid.

Since Prodigiosin has a notable antibacterial activity against these pathogenic organisms, it can therefore, be effectively used to treat disease conditions arising from these pathogenic microorganisms.

Acknowledgement

Authors are thankful to Department of Biotechnology, Smt. Chandibai Himathmal Mansukhani College, Ulhasnagar, District Thane, Maharashtra, India

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