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ORIGINAL ARTICLE **OPEN ACCESS**

Production of biopigment "Prodigiosin" From *Serratia sp.* under optimized conditions and its applications

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ABSTRACT
Prodigiosin, a biopigment, is a secondary metabolite that is a natural red-colored pigment that belongs to the family prodiginines.It has a tripyrole in its structure and is produced by many strains of the bacterium Serratia spp and other gram-negative organisms. Prodigiosin has a variety of biological activities such as antimicrobial, antifungal, antioxidant, anti-proliferativeactivity and also has wide application in textile, printing, food, cosmetic and therapeutic industries. Therefore, the present study was aimed to screen different natural samples (soil, water, and surface swab) for isolation of the prodigiosin producing Serratia spp. Isolates were identified by standard microbiological procedures and production of pigment was carried out on Nutrient broth. Extraction was carried out using acidified ethanol and subjected to spectrum scanning in range between 300-700nm using ethanol as blank followed by Presumptive test to confirm pigment as Prodigiosin. Various parameters were optimized for highest pigment production. Pigment was used to check Bio-emulsification activity, Antioxidant activity, Antimicrobial activity and Antifouling activity. In the current study, highest pigmented bacterial isolate obtained from effluent sample and thus was used for pigment production, extraction and for further studies. The highest absorption peak was observed at 530nm using ethanol as blank and pigment production was found to influence by various condition that used for optimization.
Key Words *Serratia, Pigment, Anti fouling activity, Anti oxidant activity, Prodigiosin.*

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INTRODUCTION

Serratiaspp is a Gram-negative bacterium, belonging to the family of *Enterobacteriaceae. The* characteristic feature of the organism is associated with color pigment. There are several species of Genus *Serratia*such as *Serratiapymuthica, Serratiaodorifera, Serratiaficaria, Serratialiquifaciens, Serratiarubidae, and Serratiafonticol.*

Natural products constitute one of the major sources of chemical diversity and potential medicinal use. Pigments produced by organisms as reminiscence of its secondary metabolism are commonly referred to as bio pigments that show a wide range of synthetic and commercial applications.[1]. Prodigiosin a red pigment produced by many strains of the bacterium like *Serratia marcescens* and some other unrelated microbial strains, such as *Vibrio psychroerythrus, Streptomyces griseoviridis* and *Hahellachejuensis* was found to exhibit antibacterial, antimycotic, immunomodulating, anti-tumor and anti-malarial properties[2].Prodigiosin is produced by *S. marcescens*in a bifurcated biosynthetic pathway where mono and bi-pyrrole precursors are obtained separately and then coupled to form the linear tripyrrole red pigment[3] during the stationary phase of bacterial growth[4] Prodigiosin ($C_{20}H_{25}N_{30}$) is produced by many strains of Serratia spp and is associated with extracellular vesicles or found in intracellular granules[5]. It is a member of the prodiginines [6].and shows a hydrophobic nature [7]. It is also responsible for cell surface hydrophobicity in various *Serratia* strains [8]. The prodigiosin group belongs to the tripyrrole family, which contains a 4-methoxy, 2-2 bipyrrole ring. Nowadays scope of commercial production of biopigments like chlorophyll, melanin, monascins, flavins and prodigiosn are increasing. The present studies focus on the importance of prodigiosin pigment and its antimicrobial activity and application in textile dyeing. from undecylprodiginine.

MATERIAL AND METHODS:

SAMPLE COLLECTION:

20g of Soil sample was collected from3 different public garbage areas in sample collecting vials for isolation of *Serratia*

Collecting Sites:-Khadki, Azam campus, Katraj- Pune

Isolation of *Serratia spp*

Soil suspension was prepared using sterile distilled water and streaked on sterile Nutrient Agar plates. The plates were kept for incubation at 37^0C for 24 hrs.

After incubation colony characters were recorded as well as Gram Staining and Motility test were performed.

Extraction of *pigment:*

The culture was spread on 5 sterile Nutrient Agar plates and incubated at room temperature for 2 days to obtain confluent growth of organism with brick red pigment. The growth was scrape- off in sterile saline and collected it in centrifuge tubes from all plates.

The cells were sedimented the at 3000 rpm for 20 minutes in centrifuge, pooled and digested the cell mass in glass test tubes by adding 1N NaOH, 2 times the volume of the cell mass, in a boiling water bath for 1hr.

The pigments were extracted from the digest in equal volume of Absolute Ethanol and centrifuged at 3000rpm for 20 min to get clear pink colored solution. Mixed it well with equal volume of Petroleum Ether, and vigorously shaken the test tubes on vortex mixer. Allowed the tubes to settle for 10 minutes.

After separation of layers the upper layer was collected in an evaporating dish. And the solvent was evaporated on boiling water bath till dry residue was obtained. The residue was recovered in 5 ml Acidified Ethanol.

The absorbance was measured at 530nm. The concentration of Prodigiosin was calculated.

Estimation of Prodigiosin

The absorption pattern over wavelength was initially checked and it was found that the adsorption maxima were at 530nm. At this wavelength the absorption values were recorded.

Isolated prodigiosin was estimated using the following formula-

$$\text{Prodigiosin unit / cell} = \frac{[OD\ 530 - (1.381 \times OD\ 620)] \times 1000}{OD\ 620}$$

OD Optimal density; OD 530 – Pigment absorbance ; OD 620 – Bacterial cell absorbance ; 1.381 - constant

Optimization of different parameters

Optimization of various parameters is necessary in order to determine the optimum condition for the maximum production of prodigiosin.

Optimization was carried out using Nutrient broth.

Effect of Carbon sources on prodigiosin production-

Different carbon sources used were Glucose, Sucrose, Fructose and Lactose.1ml overnight culture of *Serratia spp.* was inoculated in Nutrient broth supplemented with 1% w/v concentration of carbon sources. Incubated at 30°C on a rotary shaker for 24-48 hours. Prodigiosin production was estimated

I. Effect of Nitrogen sources on prodigiosin production-

Organic and inorganic nitrogen sources were used which included Tryptone, Yeast extract, Ammonium Chloride, Ammonium Sulfate.1ml overnight culture of *Serratia spp*. was inoculated in Nutrient broth supplemented with 0.5% w/v concentration of nitrogen sources. Incubated at 30°C on a rotary shaker for 24-48 hours. Prodigiosin production was estimated.

Effect of Salt Concentration on prodigiosin production-

1ml overnight culture of *Serratia spp*. was inoculated in Nutrient broth with 0.5%, 1%, 2%, 4% and 6% w/v NaCl concentration. Incubated at 30°C on a rotary shaker for 24-48 hours. Prodigiosin production was estimated.

Effect of pH on prodigiosin production-

pH plays an important role in maintaining an environment for the microorganism to grow luxuriantly.1ml of overnight culture *Serratia spp*. Was inoculated in Nutrient broth having different pH such as 3,5,7,9 and 11. Incubated at 30°C on rotary shaker. Pigment production was estimated.

Effect of Incubation time on prodigiosin production-

1ml overnight culture of *Serratia spp*. was inoculated in Nutrient broth. Incubated at 30°C on rotary shaker.Pigment production was estimated at intervals of 24, 48, 72 and 96 hours.

Effect of Agitation on prodigiosin production

1ml overnight culture of *Serratia spp*. was inoculated in two different flasks containing Nutrient broth. One flask was incubated at 30°C on rotary shaker while another flask was kept in static condition. Incubated at 30°C on rotary shaker for 24-48 hours. Pigment production was estimated.

Effect on Temperature on prodigiosin production-

Temperature influences the growth of microorganisms and also pigment production.1ml overnight culture of *Serratia spp*. was inoculated in different flasks containing Nutrient broth. Incubated at 25°C, 30°C, 37°C and 45°C for 24-48 hours. Pigment production was estimated.

Effect of various Natural sources on prodigiosin production-

1. 1ml overnight culture of *Serratia spp*. was inoculated in Sesame powder, Peanut powder and Peanut oil, Sesame oil containing Nutrient broth.

2. Incubated at 30°C for 24-48 hours.
3. Pigment production was estimated.

Applications studies of Pigment

Antifungal activity of Prodigiosin –

To examine the antifungal effect Disc Diffusion method was used. *Aspergillus oryza.*
was swabbed on Mueller Hinton agar plates and disc made up of fabric linen was dipped in prodigiosin and was placed on the plate.

2.6.2. Antibacterial activity of Prodigiosin –

To examine the antibacterial effect Disc Diffusion method was used. The clinical isolates were swabbed on Mueller Hinton agar plates and disc made up of fabric linen was dipped in prodigiosin and was placed on the plate.The clinical isolates used were *E.coli, Staphylococcus aureus, Pseudomonas aeruginosa, and Salmonella typhi.*

Bio-emulsification activity of *Serratia spp*-

24, hours old culture of *Serratia spp*. was inoculated in 100 ml Nutrient broth and incubated at 30◦C.After incubation, the broth was centrifuge and the supernatant was further used to check emulsifier activity. To measure emulsifier activity,3ml of kerosene, toluene, peanut oil and coconut oil was taken in different tubes.2ml of supernatant added to each tube. Tubes were vortexed at high speed for 2-5 mins.Tubes were kept in refrigerator and measurements were made 24 hours later. The emulsion index is the Height of emulsion layer, divided by the Total Height, multiplied by 100.

2.6.4 Antioxidant activity of prodigiosin-

Antioxidant is the substance that prevents the oxidation of other molecules. The total antioxidant capacity of the pigment extract was evaluated by Phosphomolybdenum method.

Phosphomolybdenum assay-

The antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation according to the method of Prieto (1999).This method is based on the reduction of phosphomolybdic acid to phosphomolybdenum blue complex by Sodium Sulfide.The obtained phosphomolybdenum blue complex is oxidized by the addition of nitrite and this causes a reduction in intensity of the blue colour.
0.1ml of the extract solution was mixed with 1ml reagent solution (6 M Sulphuric acid, 28mM Sodium Phosphate and 4mM Ammonium Molybdate).The reaction was incubated at 95◦C for 90 mins in water bath. Absorbance of solution was measured Spectrophotometrically at 695 nm using a blank.Blank was prepared by using 0.1 ml of solvent used for extraction and mixed with 1ml reagent solution and treated in the same way as above. Ascorbic acid (100µg/ml) was used as standard. The antioxidant capacity of the extract was evaluated as equivalents of ascorbic acid (µg AE/ml extract).

2.6.5 Antifouling Activity of prodigiosin

Biofilm development

The formations of biofilm was studied under laboratory conditions. The biofouling organisms used were *Staphylococcus aureus*, *Escherichia coli*, and clinical isolates of *Pseudomonas aeruginosa* obtained from pus culture. Seawater was sterilized and 500ml was poured into 3 separate beakers. Number of cells of indicator organism were adjusted to 2.8×10^8cells /ml which was confirmed by obtaining OD values using colorimeter at 600nm.1ml of each indicator organism was inoculated in the corresponding beaker.

Preparations Of Biofilm Substrate and Bacterial Enumeration:

The substrate used for testing biofilm was taken to be stainless steel sheets and wooden blocks. These substrate were sterilized at 121°C. the dimensions of stainless sheet and wooden blocks was 2"×2" .The methanol extract of the pigment was added with 1ml linseed oil which acts as a pigment blinder. The pigment was applied onto the steel and wood pieces and left for drying. Control for steel and wood was prepared without applying pigment. Methanol added with linseed oil was applied onto wood and steel to compare the effect of methanol and pigment on antifouling activity.3wooden pieces (control pigment applied wood and ethanol applied wood)and three stainless steel pieces(control, pigment applied steel and methanol applied steel) were immersed into each of the beakers containing sea water. The set up was left at room temperature for a period of 7days for formation of biofilm.at the end of 7 days, the samples were taken from the sea water and viable bacterial count was performed.The biofilm from wood and steel pieces were scraped off using a sterile spatula into 100ml of sterile saline to make master dilution for each sample, for each substrate and for each organism ,serial dilution was done in sterile saline tube.10-

fold dilution was performed by adding 1ml from aster dilution to 9ml of sterile saline. The dilution was done upto 10^5.Bacterial enumeration was done by spread plate method .NA plates were prepared and 0.1 ml of sample from dilution $10^{-4}$and 10^{-5} were spread using flame sterilized glass rod. This procedure was followed for each organism, each substrate, and for each sample. All plates were incubated at 37◦C for 24 hrs. Enumeration of viable organism was carried and the average bacterial counts were recorded. The experiments were conducted in duplicates.
Number of cfu/ of master dilution =no of colonies counted ×dilution factor /volume of sample.

RESULTS AND DISCUSSIONS

Biopigment obtained are the natural sources of pigment and have a wide applications in many field. Prodigiosin obtained from*Serratia spp*.is a red pigment which also exhibits variety of bioactive along with its application .

Isolation and identification of *Serratia spp*.**-**

Differnt natural samples were collected and screened which include 4 soil sample. Out of thatonly one sample screened for prodigiosin production. Isolate were identified by morphological, colonial and biochemical characterization as *Serratia spp*.

Table 1 – Biochemical charatcterization of *Serritia marcscens*

Sr. No	Biochemical reaction	Results
1.	Gram staining	**-**
2.	Shape	**Rod**
3.	Oxidase	**-**
4.	Catalase	**+**
5.	Indole	**-**
6.	Voges-proskauer	**+**
7.	Motility	**Motile**
8.	Citrate	**+**

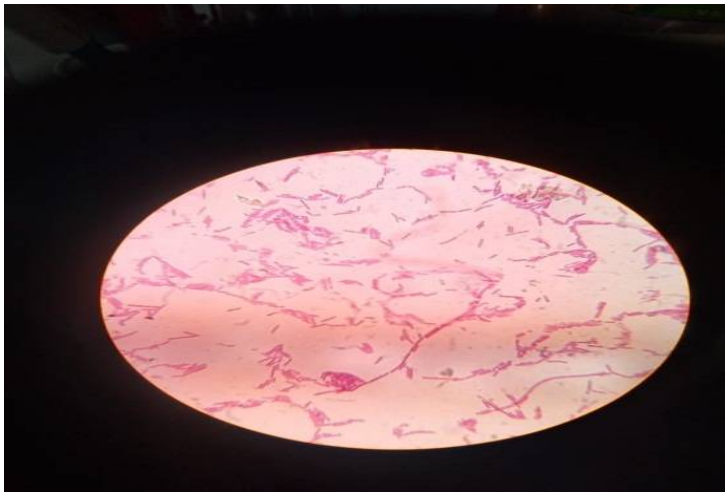

Fig No 2 :Gram staining (Gram –ve rods)

Selection of media for maximum pigment production by *Serriatia marcescens*

Among all the different media used for optimization nutrient broth suppliemented with milk was found to be best media for the maximum pigment production by *Serratia spp*.

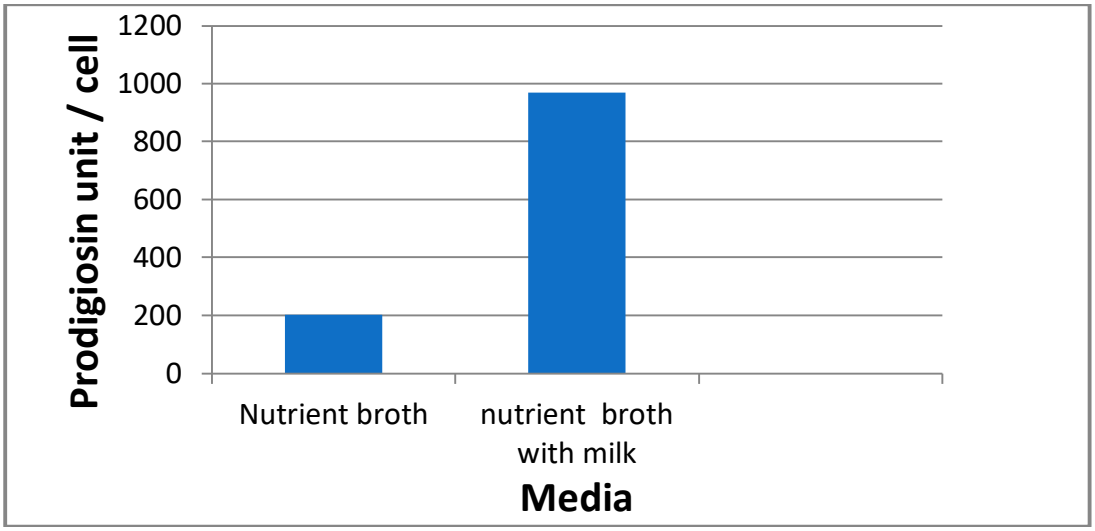

Fig 3 : optimization of Medium

Optimization of different parameter

Effect of Carbon source on prodigiosin production:-

Different carbon sources were used among which maximum prodigiosin production was observed in NB prodigiosin supplement with Glucose, Sucrose, lactose , Maltose. NB Supplemented with lactose that *Serratia spp*. showed highest pigmentation with carbon source in the medium.

According to *Prasad, M. P 2015* found that *Serratia spp*. showed highest pigmentation with dextrose as carbon source in medium.

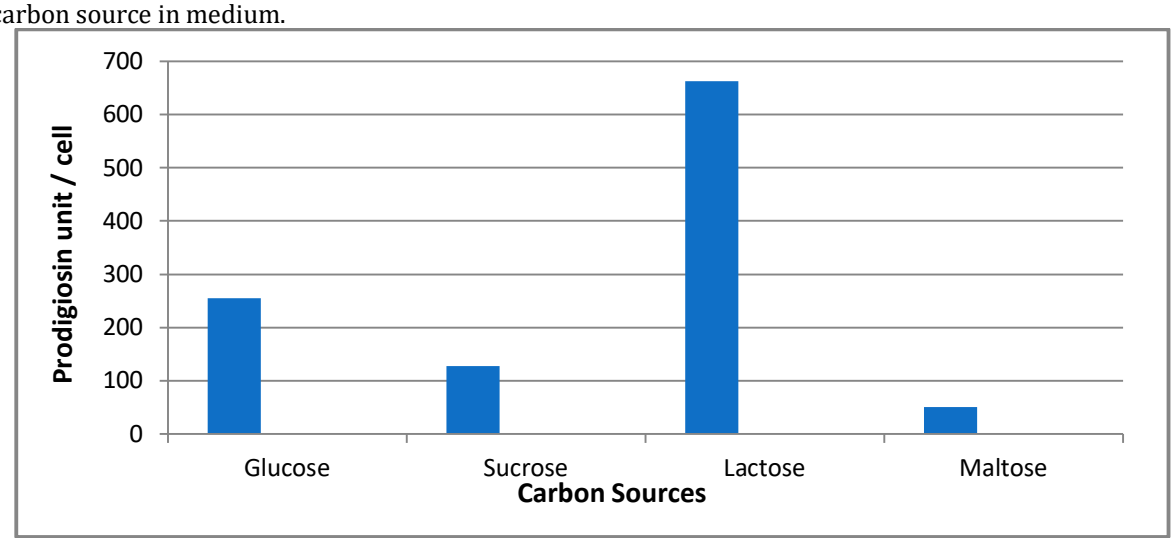

Fig 4: optimization of carbon source

II. Effect of salt concentration on prodigiosin production :-

Different concentration of Nacl Salt (0.5% , 1% , 2% , 4% , 6%) was used for the optimization . Maximum prodigiosin production was observed at 1% salt concentration while very low concentration was observed at 6% salt concentration.

According to *Samyuktha and Sayaliet.al 2016* showed that among their 3 isolates maximum pigment production was observed at 4 % salt concentration, for isolate1-4% ,for isolate 2-8% and for isolate 3-0.5%.

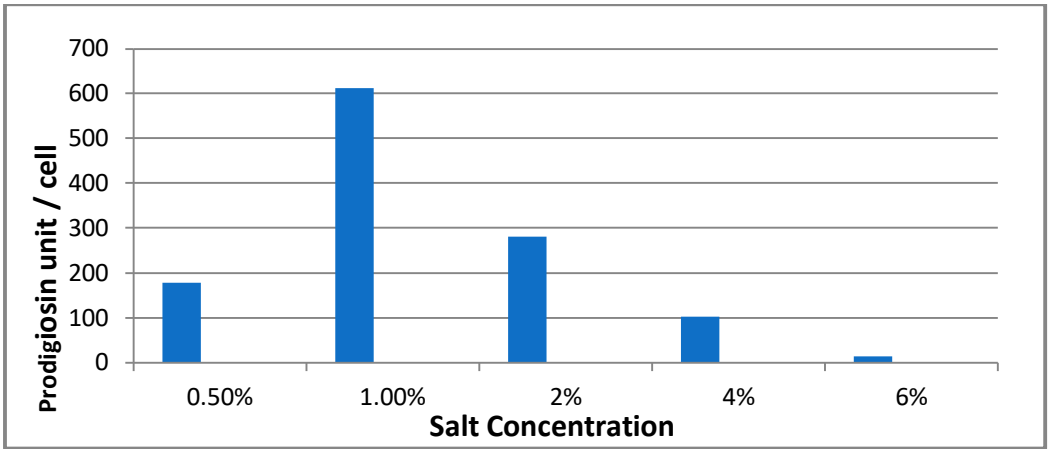

Fig 5: Optimization of salt concentration

Effect of pH on prodigiosin production:-

Medium was adjusted to different pH (3 , 5, 7 , 8 , 11). Maximum prodigiosin production was observed at pH 7 (918.36). The pigment prodigiosin was also observed in pH 3 , 5 , 8 , 11 but at very low concentration. According to *Prasad, M.P 2015* found that *Serratia spp*. showed optimum growth and pigmentation at pH7. Our results matched with their results .

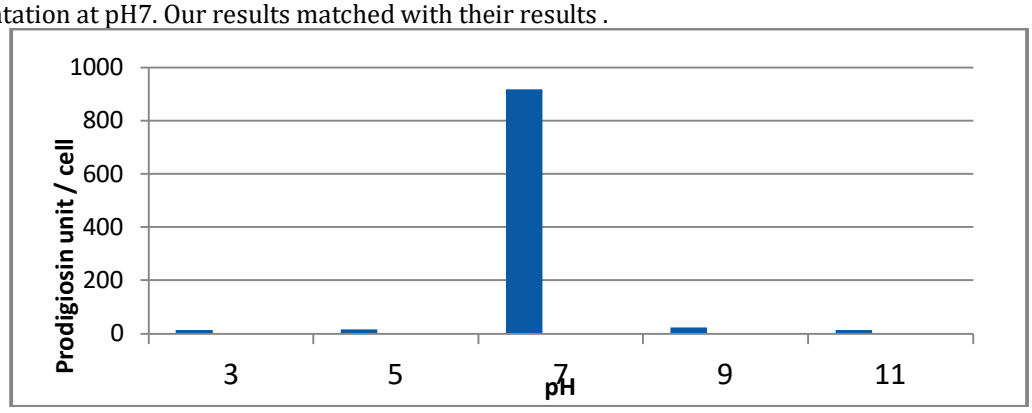

Fig 6: Optimization of pH

Effect of Nitrogen Source on prodigiosin production:-

Organic and Inorganic nitrogen sources were used for optimization. Maximum prodigiosin production was observed with Tryptone(1020.4) followed by yeast extraction ammonium chloride, ammonium sulfate (Fig).

According to *Prasad, M.P 2015* found that *Serratia spp*. showed highest pigmentation with beef extract as nitrogen source in medium.

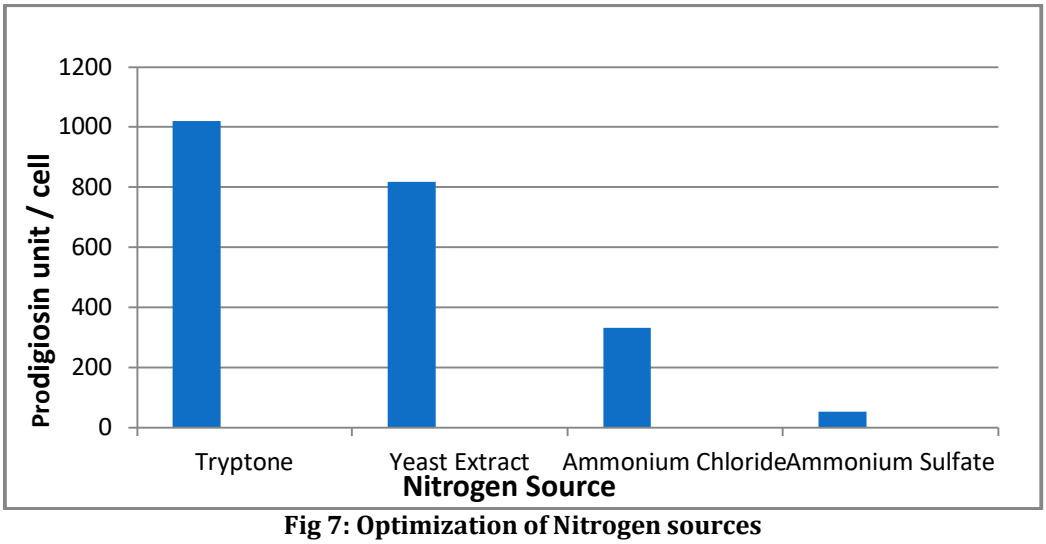

Fig 7: Optimization of Nitrogen sources

Effect of Incubation time on prodigiosin production: -

Production medium was incubated for different time periods. Maximum prodigiosin production was observed after 96 hours (549.18) of incubation time period (Fig) . Increase pigment production was observed as there was increase in incubation time.

According to Jose *et al., 2017* observed that increased pigment production was observed until 48 hours and reduces afterwards.

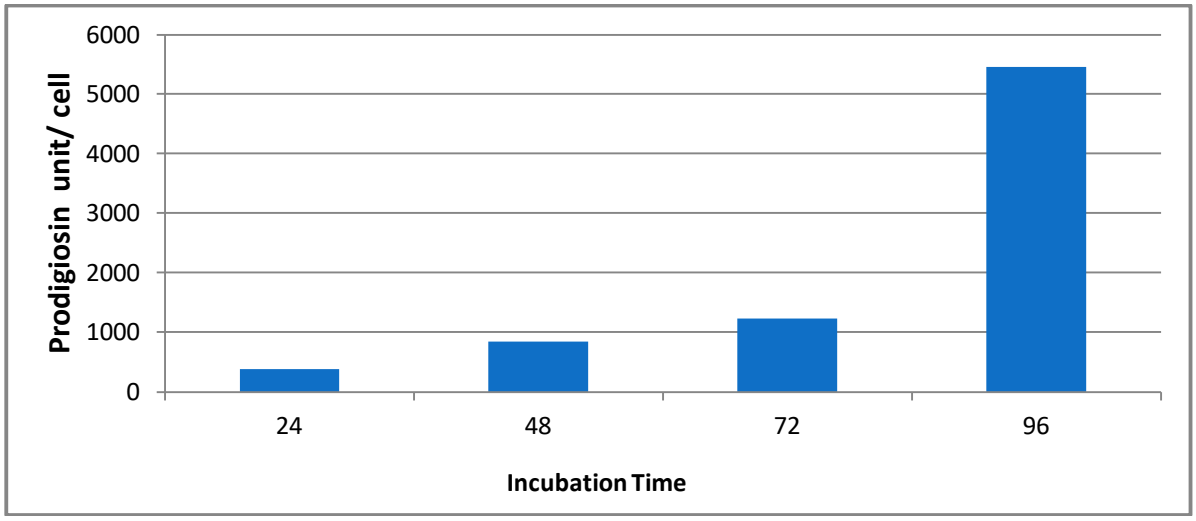

Fig 8: Optimization ofIncubation time

Effect of Temperature on prodigiosin production:-

Maximum prodigiosin production was observed at 30^{0}C (969.38) followed by 37^{0}C.

According to *Jose et al.2017* , determine that optimum temperature for pigment production is 28^0 C and 37^0 C and he observed that maximum prodigiosin production was obtain at 28^{0}C while only slight amount was obtained at 37^{0}C.

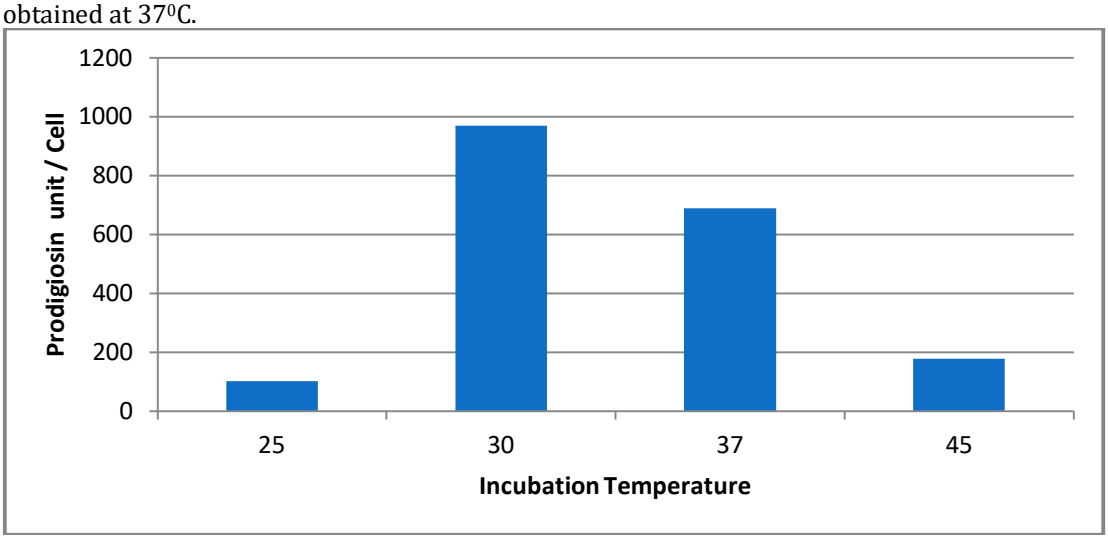

Fig 9: Optimization of Incubation Temperature

Effect of Agitation on prodigiosin production:-

Maximum prodigiosin production was observed with agitation condition (816.32) rather than static condition (fig).

According to *Jose et al., 2017* found that maximum pigment production was observed in agitation condition rather than static condition. Our results matched with their result.

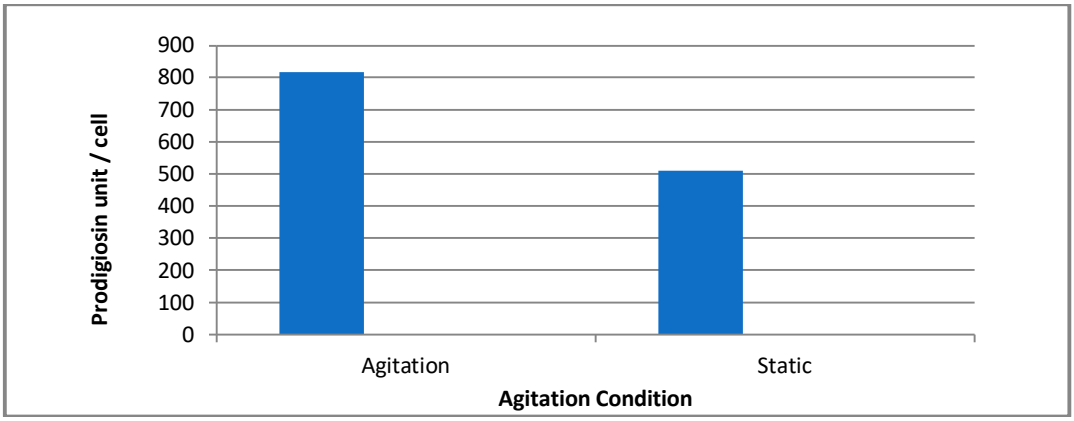

Fig 10: Optimization of Agitation Conditions

Effect of various natural sources on prodigiosin production

For selection of substrate of suitable substrate four different natural substrate have been tested namely sesame powder, peanut powder, sesame oil and peanut oil . The highest production was seen in peanut powder as a substrate (fig) the oils are known for their high levels saturated fatty acids. The media containing the fatty acid seeds gives highest production than oils.

According to *Anuradha et al*. pigment yield was 15 times more in media containing fatty acid seeds than oil . Our results matched with their results

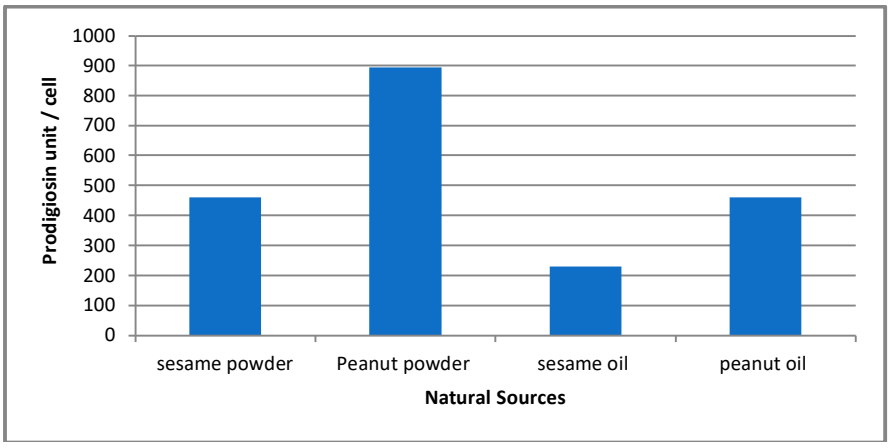

Fig11: Optimization of Natural Sources

Antifungal activity ofprodigiosin production –

The extracted pigment from *Serratia spp*. was assayed against the fungal species.*AspergillusOryza.* From this assay *Aspergillusoryzae* shows the zone of inhibition.

According to *Srimathi R, Priya R, et.al* (2017) the prodigiosin pigment showed the antifungal activity against Aspergillusflavus, Goetrichum spp. and Candida albicans .

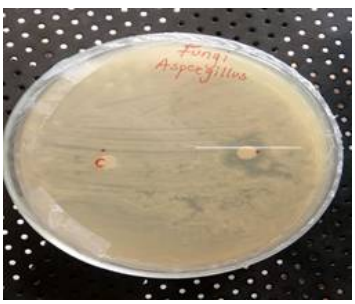

Fig No.12: Zone of inhibition *Aspergillus oryza*

Antibacterial activity of prodigiosin-

Antibacterial activity of prodigiosin pigment extracted from *Serratia spp*. was assayed against bacterial strains such as *Staphylococcus aureus, Escherichia coli, Salmonella spp., Pseudomonas spp.* From this assay

Staphylococcus aureus, Escherichia coli, Salmonella spp. showed the zone of inhibition whereas pseudomonas lack the zone of inhibition.
According to Srimathi *R, Priya R, et.al* (2017) the prodigiosin pigment showed the antibacterial activity against bacterial strains such as *Staphylococcus aureus, Escherichia coli, Salmonella spp.* According to them *Pseudomonas spp.* also showed zone of inhibition.

Fig No.13 Zone of inhibition *Salmonella spp.*

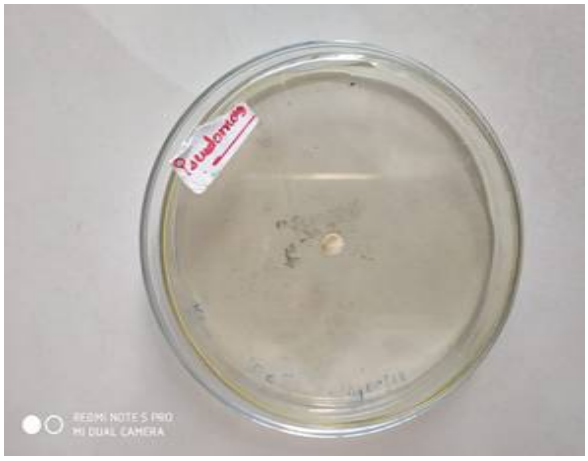

Fig No14. No Zone of inhibition *Psuedomonas spp.*

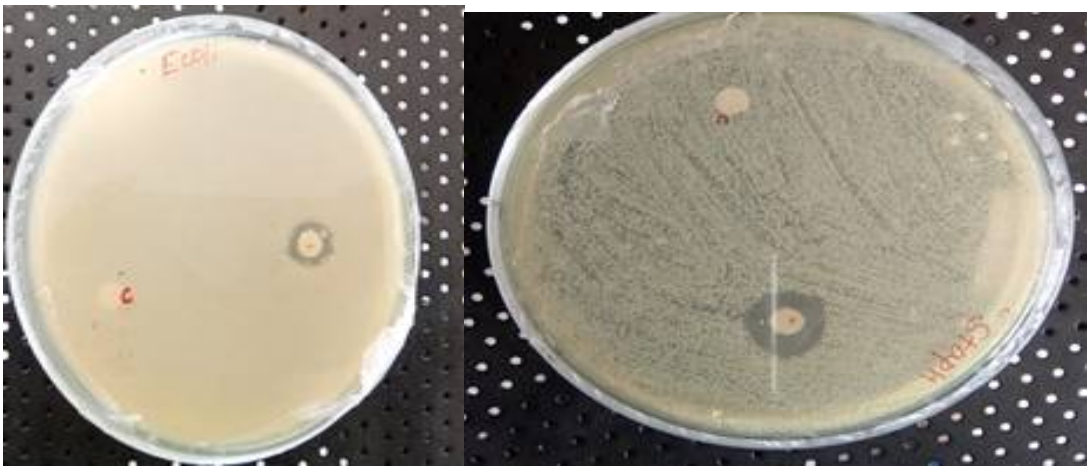

Fig No.15.Zone of inhibition *E.coli & Staphylococcus spp.*

Bioemulsification of Prodigiosin:-
Bio-emulsification activity with different hydrocarbon and oil was checked. Highest emulsification activity was observed with kerosene.

Table No. 2: Bio-emulsification activity

Hydrocarbons /Oil	E_{24}	E_{48}
Toluene	40.54	42.06
Kerosene	40.00	42.10
Coconut oil	33.33	36.11
Peanut oil	40.00	41.66

According to *Haryali H.Sorathiya , et.al 2016* highest bio-emulsification activity was observed with toluene.

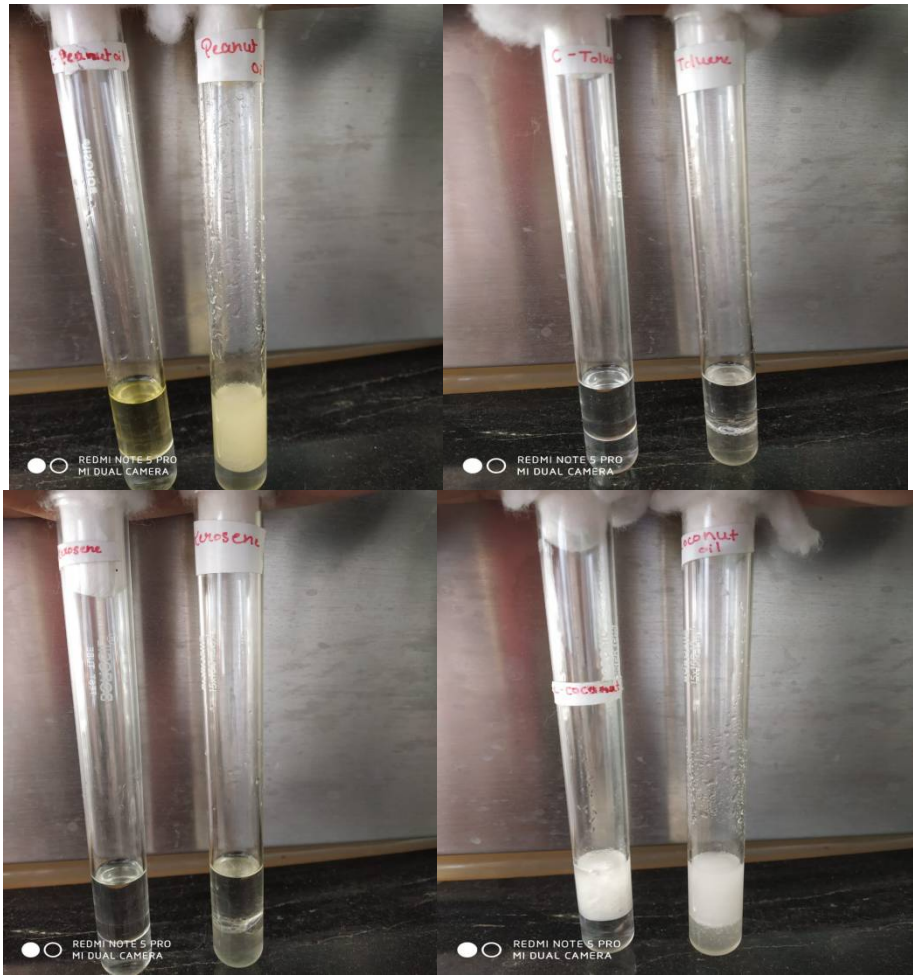

Fig No16:Bio-emulsification Activity of pigment by using Toluene, Kerosene, Peanut oil, coconut oil

Antioxidant activity of prodigiosin-

The total antioxidant capacity of the pigment extract was 30.0 µg AE/ml. The antioxidant activity of the extract was obtained lower as compared to that obtained By *Hariyali. HSorathiyaet.al 2016* which was 200 µg AE/ml.

Fig No 17. : Antioxidant activity of pigment Prodigiosin

Antifouling activity of prodigiosin production-

Estimation of viable count of the fouling organisms

At the end of the 7th day, the substrates were removed from the marine water and enumeration of bacteria was done. The number of colonies in pigmented-coated substrates were counted in the plates and compared with those in control and methanol-coated substrates. There was a difference in the

number of colonies formed in controls, and pigment-coated wood and steel. The experimental setup for biofilm formation has been depicted in Figure 1Paired student s T-test was used to analyze and compare the data for significant differences in the mean values of cell numbers. There was a significant difference in the reduction of colony-forming units in pigment-coated substrates when compared to controls. The control and Pigment coated substrates could be considered one and the same as the mean values showed no significant differences. The pigment-coated wood and steel were found to significantly reduce the adhesion of the biofouling organisms. This has been depicted in the two-tailed T- test performed. This shows that the effect of pigment application has reduced the number of biofilm-forming bacteria considerably.

In comparison with wood, the organisms have less adhesion towards steel. This was shown by the reduction in colonies in the steel by a factor of 10. The possible reason could be that the surface of steel is smoother than that of wood. It could be concluded from the statistical analysis that it is the action of the pigment that inhibited the attachment of biofilm-forming bacteria rather than methanol. It can also be inferred that the pigment prodigiosin has inhibitory activity against the marine fouling pathogenic organisms.

CONCLUSION

Based on the study, an attempt was carried out to isolate the pigment-producing*Serratia spp*. from the soil. The highest yield of the pigment was obtained from the Nutrient broth with milk but peanut seed medium was found to be the best and the cheapest source for the prodigiosin pigment production from the *Serratia spp*. The pigment has antimicrobial activity. In large-scaleproduction, the pigment will make it an alternative to chemical dyes and it can also be used as an approach to combat bacterial and fungal infections.

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