ROLE OF PIGMENT IN THE STRESS RESPONSE OF *SERRATIA RUBIDAEA* ISOLATED FROM *ANNONA RETICULATA*

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Abstract Microorganisms are known to produce a variety of pigments. Microbial pigments find several applications and are thus of commercial importance. Studies on the response of microbes to environmental stressors form a thrust area of contemporary biological research. Cells that are exposed to stress undergo changes in their metabolism to adapt to changes in their environment. In the present work, a pigmented culture isolated from a spoiled portion of a *Annona reticulata* (Custard apple) was used to study the effect of some stress factors on pigmentation. The isolate was identified as *Serratia rubidaea* based on morphological, cultural, biochemical and molecular characterization. Effect of salt, pH, temperature, heavy metal stress and growth under reduced oxygen tension on pigmentation of the culture was studied. Salt was found to inhibit pigmentation at 8% concentration. Low and high temperature of the growth range was found to inhibit pigmentation and good pigmentation was observed over a range of 15°C to 35°C. Pigmentation declined under alkaline conditions and interestingly was enhanced under slightly acidic and alkaline pH. Growth of the culture took place in presence of Cadmium Chloride up to 1 mg/ml but pigmentation was inhibited at 0.5mg/ml concentration and the culture could grow at 3mg/ml of Lead Acetate but pigment synthesis was lost at 2mg/ml concentration. Thus, the results obtained with the two salts point to the effect of heavy metal stress on pigment synthesis. Growth under low oxygen tension interestingly enhanced pigment synthesis. Hence, the study indicates that pigmentation is influenced by extremes of temperature of growth, alkaline pH and high salt and heavy metal concentration in the medium.

Keywords*: Microbial pigments, salt stress, temperature stress, pH stress, heavy metal stress, Cadmium Chloride stress, Lead Acetate stress, spoilage.*

Introduction: Pigments are chemical compounds that absorb light in the visible region [**1**]. Pigments can be classified as natural, synthetic, or inorganic. Natural pigments are produced by organisms such as plants, animals, fungi, and microorganisms. Natural and synthetic pigments are organic compounds. Inorganic pigments can be found in nature or reproduced by synthesis [**2**]. Some examples of naturally occurring pigments are Riboflavin, Beta-carotene, Canthaxanthin, Carotenoids, Prodigiosin, Phycocyanin, Violacein, Astaxanthin [**3**]. Pigments come in a wide variety of colors, some of which are water-soluble. Some natural functions proposed for microbial pigments are protection against ultraviolet radiation, protection against oxidants, protection against extremes of heat and cold, protection against natural antimicrobial compounds produced by other microbes, antimicrobial activities against other microbes, acquisition of nutrients, such as iron, acquisition of energy by photosynthesis (e.g. *Cyanobacteria)* [**4**].

Pigments are of importance to many industries.

In the food industry they are used as additives, color intensifiers, antioxidants, etc. [5].

Red-pigmented prodigiosin compounds were first isolated from *Serratia marcescens* and identified as secondary metabolites. The common aromatic chemical structure of these pigmented compounds was first named prodiginine by Gerber [6]. Prodigiosin was the first prodiginine for which the chemical structure was determined. Prodiginines share a common pyrrolyldipyrromethene core structure and have a wide variety of biological properties, including antibacterial, antifungal, antimalarial, antibiotic, immunosuppressive, and anticancer activities. Such properties have made them one of the most powerful research tools in the past decade [7].

The environment is becoming increasingly stressful. Cells that are exposed to stress such as salinity, osmotic pressure, drought, heat, radiations and other factors undergo changes in their metabolism in order to adapt to changes in their environment. Stress result in changes in the morphological, physiological and biochemical responses and adversely affects the growth and development of cells [8].

Hardjito *et al.* (2002) studied the influence of environmental conditions on the production of pigment by *Serratia marcescens*. They isolated *Serratia marcescens* biovar A2/A6, from an Indonesian freshwater source. Formation of pigment was found to be strongly influenced by environmental conditions. The optimum conditions for growth and pigment formation were found to be a temperature of 30°C in a neutral to slightly alkaline medium containing lactic acid and beef extract [9].

Yamazaki *et al.* (2006) studied the effect of salt stress on pigment production of *Serratia rubidaea* N-1 isolated from the Ariake Sea, Japan with an aim to study the usefulness of the strain as an indicator bacterium for the detection of quorum sensing inhibitors (QSIs) from marine microbes. A large amount of prodigiosin was produced and released from the cells into the culture medium containing 1M NaCl. The pigment production started at the late logarithmic phase of growth, increased gradually, and reached the maximum level at the stationary phase after 24 h of incubation. A higher-level of pigment production was observed at 0.8 M NaCl when the cells were incubated for 48hrs in Luria-Bertani broth medium . From these results, it appears that *S. rubidaea* N-1 belongs to a salt-tolerant bacterium of which prodigiosin is produced dependent on the salt concentration [10].

Stress results due to exposure to DNA damaging agents. The stress factors include nutritional depletions, temperature shift, osmotic pressure, micro-aerobic conditions, presence of heavy metal ions, and microwave irradiation. At the molecular level, in particular, up-regulation and activation of error-prone DNA polymerases, down-regulation of error-correcting enzymes, and movement of mobile genetic elements are common features of several stress responses leading to genetic changes. The reports on effect of stress on pigment production are scanty and therefore, study was undertaken to examine the effect of the various factors on bacterial pigmentation and evaluate difference between them using a pigmented culture isolate as a model [11]. The importance of the study is to understand the response of bacteria to stress with respect to pigmentation that will help optimize conditions for best pigment production for commercial synthesis.

Materials and Methods:

Isolation and Maintenance of Cultures from Food Samples:

Custard apple showing spoilage was used for the isolation of pigmented organisms. A homogenized suspension prepared in saline was

used for the isolation by streaking on sterile Nutrient Agar plates. Incubation was done at 30°C for 48 hours. A single type of pigmented colony was obtained. A well isolated colony was sub cultured on sterile nutrient agar slant. The isolate was further purified and the pure culture was streaked on sterile Nutrient agar slants in triplicate. One was used as working stock and the other two were preserved at 4°C. Slant cultures were sub-cultured once a month.

Morphological Characterization:

The bacterial isolate was analysed at macroscopic level by general morphological description of the colony such as elevation, form, and margin [**12**]. The morphology, arrangement and Gram character were also determined by examination under oil immersion objective.

Biochemical Characterization:

Biochemical characterization of the bacterial isolate was done using the following sterile media: Peptone water base with 1% of sugars (glucose, glycerol, galactose, mannose, lactose, xylose, arabinose, maltose, ribose, sucrose, mannitol), Nitrate peptone water, sterile Nutrient broth with salt at different concentrations, Nutrient broth prepared in buffer with different pH values , Simmon's citrate agar slant, Christensen's urea broth, Tryptone water, Triple sugar iron agar, 2% Peptone water with Lead acetate paper, Phenylalanine agar, NB with 10% and 40% bile salt and Motility agar. Reagents used: α-Naphthylamine, Sulfanilic acid, Kovac's reagent, 10% ferric chloride aqueous solution, 3% Hydrogen peroxide and Tetra methyl paraphenylene diamine dihydrochloride reagent. Culture identification was done by referring to Bergey's manual of determinative bacteriology [**13**].

Determination of 16S rRNA sequence: Molecular identification of the isolate was done by sequencing its 16 S rRNA gene. 16S rRNA sequence was done at "Chromous Biotech Pvt. Ltd", Bangalore.

Stress Response Study:

Response to Salt Stress:

Standardized culture suspension of the isolate (10^8 cells/ml) was used for spot inoculation on sterile Nutrient agar plates having 2%, 4% 6%, 8%, 10%, 12%, 14%, 16%, 18%, 20% salt and incubated at 30°C for 48hrs [**14**].

Response to Low and High Temperature Stress:

Standardized culture suspension of the isolate (10^8 cells/ml) was used for spot inoculation on sterile Nutrient agar plates and incubated at various temperatures (8°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C) for 48hrs [**15**].

Response to pH Stress:

5ml Nutrient Broth tubes with pH in the range of 4 to 10 were prepared. 0.5ml of the culture suspension was inoculated in sterile NB tubes with pH 4 to 10 and incubated at 30ºC for 48 hrs [**15**].

Response to Heavy metal Stress (Cadmium and Lead):

Determination of Maximum tolerance concentration (MTC) by Tube Method: Double dilution in sterile Nutrient Broth: Standardized culture suspension of the isolate (0.5ml) was used for inoculation in sterile Nutrient Broth tubes with different concentrations of the heavy metal ions. Results were recorded after 48 hrs of incubation at 30°C.

Effect of Heavy metal salts on pigment production:

Based on the results of the tube assay, plate assay was designed to evaluate the effect of Cadmium and Lead ions on pigment synthesis.

Lead ions: Sterile Nutrient agar plates having concentration of 0.5, 1, 2 & 3 mg/ml were

prepared. Standardized culture suspension of the isolate**s** (10^8cells/ml) was spot inoculated. The plates were incubated at 30°C and observed for pigmentation after 48 hrs.

of the isolate (10^8cells/ml) was spot inoculated. The plates were incubated at 30°C and observed for pigmentation after 48 hrs.

Growth under Low Oxygen Tension:
Standardized culture suspension of the isolate (10^8 cells / ml) was used for spot inoculation on sterile Nutrient agar plates and incubated in a Candle jar under reduced oxygen tension at 30°C incubation. Results were recorded after 48 hrs. of incubation.

Results and Discussion: Spoilt portion from the custard apple sample was homogenised and the suspension upon isolation on sterile Nutrient agar plate gave red coloured colonies. The isolate was purified and subcultured on sterile Nutrient agar slant in triplicate for further work. It was coded as Isolate 1.

Morphological Characterization: Colonies of the isolate on Nutrient Agar were red, circular, mucoid, slightly raised and 1mm in diameter. They were observed to be short Gram negative rods upon Gram staining.

Biochemical Characterization: Biochemical tests were carried out and from the results obtained, the isolate was found catalase-positive, oxidase-negative, able to tolerate salt up to 5% concentration (w/v), grow over a pH range of 6.5-9.6, urease negative, Nitrate reductase positive and ferment all the sugars tested. The results of biochemical tests performed are shown in **Table 1.**

Table 1: Biochemical results

Test	Isolate I
Sugar Fermentation tests:	+
Glucose	
Lactose	+
Glycerol	+
Galactose	+
Mannose	+
Xylose	+
Arabinose	+
Maltose	+
Ribose	+
Mannitol	+
Nitrate Reductase test	+
Growth at - 5% NaCl	+
7.5% NaCl	-
10% NaCl	-
12% NaCl	-
18% NaCl	-
Growth at - pH 4.8	-
pH 6.5	+
pH 9.6	+
Citrate Utilization test	+
Urease	-
Indol production	-
Growth on TSI – slant	Alkaline
Butt	Acid
Gas	Nil
H_2S	-
Desulfurase test	-
Phenyl Alanine Deaminase test	-
Bile solubility	
i. 10% bile salt	+
ii. 40% bile salt	+
Oxidase	-
Catalase	+

Cadmium ions: Sterile Nutrient agar plates having concentrations of 0.05, 0.5, 1, 2 & 3 mg/ml were prepared. Standardized culture suspension

Determination of 16S rRNA sequence: 16S ribosomal RNA (rRNA) sequencing is a common amplicon sequencing method used to identify and compare bacteria present within a given sample. Data from 16S studies are used to improve the sensitivity and specificity of taxonomic assignments, down to the species level. The sequence determined for the isolate is as shown in Box 1. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold. Based on morphological, cultural and biochemical characters, the culture isolate was identified as a *Serratia* species as per Bergey's Manual. The 16S rRNA gene sequence of isolate was analyzed by the BLAST program in NCBI Blast and was identified as a strain *Serratia rubidaea*.

Box 1. Fasta Sequence for the Isolate

AGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGAGCTTAACTTGGGAACTGCATTTG
AAACTGGCAAGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGA
GATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGT
TGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGC
AAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
TGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGGATTGGTGCCTT
CGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTC
CCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCC
AGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACA
CACGTGCTACAATGGCGTATACAAAGAGAAGCGAACCTGCGAAGGCAAGCGGACCTCATAAGTAC
GTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATC
AGAATGCTACGGTGAATACGTTCCC

Effect of Stress factors:

Effect of Salt Concentration on Growth and Pigmentation: Environmental abiotic and biotic stress factors have been proven to effect variety of responses in microbes. Some microorganisms develop systems to counteract the effect of osmotic stress such as salt stress (NaCl) [**16**]. The results for salt stress on growth and pigmentation are shown in **Table 2** and **Figure 1**. *Serratia rubidaea* strain could tolerate salt upto 8% but pigment was inhibited. Similar results were obtained by **Yamazaki *et al.*** [**10**]**.** They studied the effect of salt stress on pigment production in *Serratia rubidaea* N-1 and observed a higher-level of pigment synthesis at 3.5% - 5.85% concentration while in the present study pigmentation range was found to be 0.5% - 4% NaCl [**10**].

Table 2: Effect of Salt Concentration on *Serratia rubidaea*

Salt Concentration	Growth	Pigment
0%	++	P
0.5%	++	P
2%	++	P
4%	++	P
6%	+	P
8%	+	Nil
10%	-	-
12%,14,16,18,20%	-	-

Key: + = Growth; – = No Growth; P = Pigment; Nil = No Pigment

Figure 1: Effect of Salt Concentration in *Serratia rubidaea*

Effect of Temperature on Growth and Pigmentation: Temperature is one of the most influential physical factors that regulate growth and other physiological phenomena of microorganisms [**17**]. The results for the effect of low and high temperature on growth and pigmentation are shown in **Table 3** and **Figure 2.** The culture showed a growth temperature range of 8°C - 40°C. Pigment synthesis was inhibited at 8°C and 40°C. Growth at lower temperatures indicates the culture is psychrotrophic and likely to be involved in the discoloration of the food articles preserved at low temperatures such as in the refrigerator. Similar results were obtained by **Goswami *et al.*** [**18**]. They studied the effect of temperature on pigment production in a bacterial isolate obtained for soil. Pigmentation was observed at temperatures between 15°C and 40°C with maximum at 30°C [**18**].

Table 3: Effect of temperature on growth and pigmentation of *Serratia rubidaea*

Temperature	Growth	Pigment
8°C	+	Nil
15°C	++	P
20°C	++	P
25°C	++	P
30°C	++	P
35°C	+	P
40°C	+	Nil
45°C, 50°C, 55°C	-	Nil

Key: + = Growth; – = No Growth; P = Pigment; Q = Decrease in intensity of pigment; Nil = No Pigment

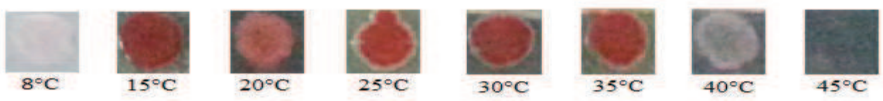

Figure 2: Effect of Temperature on Growth and Pigmentation of *Serratia rubidaea*

Effect of pH on Growth and Pigmentation: The results for the effect of pH on pigment production are presented in **Table 4.** No pigment was made by the cells at pH 10. Under alkaline pH, pigmentation property declines while at acidic pH and slightly alkaline pH, pigmentation is enhanced. Similar results were found by **Goswami *et al* [18].** They studied the effect of pH on pigment production in a bacterial isolate obtained from soil. Considerable growth and pigmentation was observed at pH between 6 and 10, with the maximum at pH 7 **[18]**.

Table 4: Effect of pH on growth and pigmentation of *Serratia rubidaea*

pH	Cell mass	Pigment
4	++	P +++
5	++	P++
6	++	P++
8	+++	P+++
9	+++	P+
10	++	Nil

Key: + = growth; P = Pigment

Effect of Heavy Metal Ions on Growth and Pigmentation: Effect of heavy metal stress on growth and pigmentation of the culture was studied in nutrient broth medium containing 0.00097657 to 0.5mg/ml $CdCl_2$ and Pb $(CH_3COOH)_2$. MTC for the two heavy metals was determined by observing turbidity in the tube after 48 hr. incubation against an uninoculated broth used as a blank. The results are shown in **Table 5.** Maximum tolerance concentration for Lead acetate was found to be 0.25 mg/ml and for Cadmium Chloride was found to be 0.0625 mg/ml.

Table 5: Growth response of the culture to Lead acetate amd Cadmium, Chloride

Concentration in mg/ml	Lead acetate	Cadmium Chloride
0.5	-	-
0.25	+	-
0.125	+	-
0.0625	+	+
0.03125	+	+
0.015625	+	+
0.0078125	+	+
0.00390625	+	+
0.00195313	+	+
0.00097657	+	+

Key: + = Growth; – = No Growth

Effect of heavy metal salts on pigment production was performed using plate assay method and the results are as shown in **Figures 3 and 4**. In case of Lead acetate, growth was observed at all the four concentrations used including 0.5, 1, 2 & 3 mg/ml. But pigmentation was lost at 3 mg/ml concentration. In case of Cadmium Chloride, growth and pigmentation was checked at 0.05, 0.5, 1, 2, and 3mg/ml concentrations. Growth was observed only up to 1mg/ml concentration and pigmentation was inhibited at 0.5 mg/ml. Thus the culture was found to be about forty times more sensitive to pigment inhibition by Cadmium Chloride as compared to Lead acetate. **Hassen *et al.*** (1998) studied the effects of heavy metals on *Pseudomonas aeruginosa* and *Bacillus thuringiensis*. They found that Mercury (0.02mM) and Cobalt (0.1mM), at concentrations below the MIC, always inhibited the synthesis of pigments in *Pseudomonas aeruginosa* **[19]**.

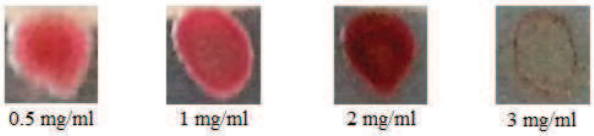

Figure 3: Effect of Lead Acetate on Pigmentation

Figure 4: Effect of Cadmium Chloride on Pigmentation

Effect of Low Oxygen tension on Growth and Pigmentation: The results for the effect of low oxygen tension on growth and pigment production are shown in **Figure 5**. Pigmentation under low oxygen tension was found to be more intense as compared to pigmentation of the culture grown under normal oxygen tension Thus growth at low Oxygen tension seems to enhance pigment synthesis in *Serratia rubidaea* culture. However, no reference could be found relating to this aspect during literature search.

Figure 5: Effect of Low Oxygen Tension on Growth and Pigmentation

Conclusion: A red pigmented isolate was obtained from a sample of spoiled custard apple and it was identified as *Serratia rubidaea* using biochemical characterization and 16S ribosomal RNA sequencing method. Response to various stress factors including salt, pH, temperature, heavy metals and growth under reduced oxygen tension were studied.

It was found that salt stress inhibits

pigmentation in *Serratia rubidaea* at a concentration of 8%. The culture showed loss of pigment at 8°C and 40°C and at pH 10. Under alkaline pH, pigmentation declines while at acidic pH and slightly alkaline pH, pigmentation is enhanced. The culture was more sensitive to Cadmium Chloride as compared to Lead Acetate with respect to both growth and pigmentation. Both the heavy metal salts arrested pigmentation of the culture at 0.5mg/ml in case of Cadmium Chloride and 3 mg/ml in case of Lead Acetate. Thus the results obtained with the two salts clearly point to the effect of heavy metal stress on pigment synthesis. Low O_2 tension, however had a positive effect on pigment synthesis as the pigmentation was enhanced. Hence, stress changes the morphological, physiological and biochemical responses and adversely affects the growth and development of cells. Thus, the present study shows that pigment production in *Serratia rubidaea*, a model organism used in the work, is influenced by the factors used in study including temperature of growth, salt concentration in the medium, presence of heavy metal ions, Hydrogen ion activity of the medium, and growth under low Oxygen tension. There may be many other factors affecting pigmentation in bacterial isolates. An understanding of the effect of these factors and regulation of biosynthetic pathways for pigment production will help to develop a controlled bioprocess for the enhanced production of the desired pigment, for suitable commercial applications.

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