EXTRACTION, CHARACTERIZATION AND OPTIMIZATION OF PIGMENT PRODUCED FROM SERRATIA MARCESCENS

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ABSTRACT
The present study was carried out to extract pigment from Serratia marcescens. The bacterium Serratia marcescens was isolated from waste water sample and characterized both morphologically and biochemically. Further identification of the Serratia sp were done by 16rRNA sequencing and confirmed to be Serratia marcescens. The pigment was extracted from Serratia marcescens by HCl acid extraction method and the sample was purified for further analysis. Purification of the pigment was done by column chromatography and the pigments were separated from the impurities by silica gel plate using methanol, ethyl acetate, chloroform, water, in the ratio of 6:3:1:1. The pink color spot was observed under UV light which confirms the pigment as prodigiosin like compound.

KEY WORDS: Serratia marcescens, Pigment, Optimization, Thin layer chromatography, Column Chromatography.

INTRODUCTION
Microbial products recently been widely used for therapeutic treatments. Such products are called secondary metabolites or bioactive compound which includes pigments, enzymes, steroids and antibiotics. Prodigiosin and prodigiosin like pigments are example for bioactive compound produced by many microorganisms. Prodigiosins are red pigment naturally occurring as (tripyrrolmethane structure) linear tripyrrolering, undecyl prodigiosin, cycloprodigiosin, meta cycloprodigison, dipyrroly dipyromethane depending on various
organisms. *Serratia sp* occurs in water, soil, plant, insects, man, and also in animals. Natural pigments can be obtained from two major sources namely plants (Mizukami et al., 1978; Papageorgiou et al., 1979) and microorganisms (Cross and Edinberry 1972; Cho et al., 2002). In this study the pigment were extracted and characterized from *Serratia marcescens* and optimization of pigments were carried out.

**MATERIALS AND METHODS**

**Isolation of “Serratia sp” Gram-negative bacteria**

The bacterial strains used in the present study were isolated from the waste water samples collected near Sitra, Coimbatore. The water samples were serially diluted from $10^{-3}$ to $10^{-8}$ and spread plate were done on nutrient agar medium and incubated at 37°C for 24 hours.

**Biochemical characterization of “Serratia sp”**

The selected bacteria were characterized by performing Gram staining, motility test, biochemical tests like Indole, Methyl red, Voges proskauver, Citrate utilization test, Nitrate reduction, Oxidase, H$_2$S tests were performed according to the procedures of Aneja (2003) and the genus of the organism was characterized as per Bergey’s Manual of Determinative Bacteriology (2003).

**Genomic DNA Isolation**

Genomic DNA was isolated from overnight culture of *Serratia marcescens*. 1.5ml of the culture cells were harvested by centrifugation at 7,500 rpm for 10 minutes and suspend in lysing buffer. Incubate at 60°C for 20-30 minutes. To this mixture add 8μl of Proteinase K and incubate at 37°C for 1 hour and cool. And add 20μl of phenol, Chlororform, Isoamly alcohol (25:24:1) and 200μl of TE buffer was added and then vortex for 3–4 minutes. Again centrifuge the suspension at 10000 rpm for 10 minutes. To the new eppendorf tube transfer the aqueous phase and adds 1ml of 95% ethanol which forms precipitation. Suspended the precipitate to TE buffer of 1μl of RNase and incubate until the pellet gets dissolves. Add 4M Ammonium acetate and add 95% ethanol, then centrifuge and the supernatant were discarded. To the pellet add TE buffer and store at 4°C. Load the DNA sample in 1% agarose gel for electrophoresis.

**Amplification by PCR**

The PCR reaction mixture contained 1μl of the DNA template and 2μl of forward and reverse primer, 1U of Tag polymerase 1.5mM of MgCl$_2$ and 200mM of each dNTP. The above
reaction mixture was subjected to an initial denaturation of 94°C for 5 minutes. Following samples were subjected to 30 cycles in PCR. The amplified fragments were then examined by running on 1.5% agarose gel and the bands were identified using ethidium bromide staining.

**Optimization of pigment production using different media**

The production of pigments from the selected organism was carried out using different media such as Trypticase soya broth, Nutrient broth, Brain heart infusion broth, Peptone Glycerol broth, Modified broth (Beef extract and yeast extract) was prepared and 1% of oil substrates were used namely oil ground nut oil, coconut oil, olive oil, gingelly oil, castor oil and sterilized at 121°C for 15 minutes at 15 lbs pressure. The inoculum was inoculated in 100ml of the broth and incubated for 48 hours at 28°C to determine the pigment production.

**Effect of pH and Temperature on pigment production**

The medium was prepared with varying pH range from 5 to 9 and incubated for 48 hours to identify the growth and pigment production. Growth was tested with different temperature ranging from 30°C, 35°C, 37°C, 45°C, and 48°C. The results were observed to determine the optimum temperature of pigment production.

**Pigment extraction**

**Acid extraction**

The cultural broth was washed off with acetone containing 10% (v/v) of 3N-HCL and extracted by rotary shaker for an hour.

**Methanol extraction**

Trypticase soya broth of 500ml containing pigment was centrifuged and the pellets were mixed with a few ml of equal volume of methanol and the mixtures were kept in rotary shaker for 20 to 30 minutes.

**Ethanol extraction**

The 48 hour culture broth containing pigment was centrifuged at 10,000rpm for 10 minutes. Cell mass (pellet) was collected and then washed for few times with 0.85% saline buffered at pH 7 with 0.01M phosphate in which the bacteria were digested by boiling with 1N NaOH for an hour in water bath. Pigment was extracted from the digest with equal volume of absolute ethanol and the extract was vortexed well.
Alkaline extraction
The cells were washed with 0.9% (w/v) NaCl and solid NaOH was added with constant agitation up to a concentration of 10% (w/v). After 2 hour, absolute ethanol and light petroleum were added in equal amount to the volume of the solution.

Purification of pigment by Column Chromatography
The crude pigment products were dissolved in 20ml of methanol and the solution was passed through a hexane-balanced silica gel column to trap the target product within the column. The size of the column used for column chromatography was (1.0×10) cm. The loaded column was eluted with 10M ethyl acetate to liberate the adsorbed product. After completion of the chromatography, the different fractions were collected at a time interval of 1 hour, and the readings were taken by colorimetrically.

TLC Analysis
TLC was carried out using ready made aluminum packed silica gel plates

Analysis of pigment
Silica gel plate was spotted with 10μl of sample containing extracted pigment and kept in a chamber containing methanol, ethyl acetate, chloroform, water. (6:3:1:1). The solvent run allowed up to 10cm and the plate was removed and air dried for 10 minutes. Faint spots were seen and fluorescence was observed under UV light. For effective separation of the impurities, the plates were dried at 45°C to obtain the purified product (red powder) (Davaraj Naveen Raj et al., 2009).

RESULTS AND DISCUSSION
In the present investigation pigment was extracted from Serratia marcescens and characterized. The bacterium used in this study was isolated, characterized and identified by 16rRNA sequencing. The growth of the pink colonies on nutrient agar revealed that the organism was found to be Gram-negative and the results were illustrated in plate 1. The bacterial morphology and species identified were confirmed to be Serratia marcescens. The morphological and biochemical characterization of the bacterium with reference to genus and species were given in Table 1. The species identification was carried out by isolating the genomic DNA. The genomic DNA was amplified and the PCR product of the sample was sequenced. The sequencing result showed that the bacterium was confirmed to be Serratia marcescens.
Table 1. Biochemical characterization of *Serratia marcescens*

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Biochemical test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Test for Genus Identification</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Motility</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>Gram Staining</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>Indole test</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>Methyl red test</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>Voges proskauer</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>Citrate utilization</td>
<td>Positive</td>
</tr>
<tr>
<td>7</td>
<td>Nitrate reduction</td>
<td>Positive</td>
</tr>
<tr>
<td>8</td>
<td>Catalase</td>
<td>Positive</td>
</tr>
<tr>
<td>9</td>
<td>Oxidase</td>
<td>Negative</td>
</tr>
<tr>
<td>10</td>
<td>Hydrogen sulfide production</td>
<td>Negative</td>
</tr>
<tr>
<td>11</td>
<td>Carbohydrate fermentation</td>
<td>Positive</td>
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<tr>
<td>12</td>
<td>Starch hydrolysis</td>
<td>Negative</td>
</tr>
<tr>
<td>13</td>
<td>Lactose hydrolysis</td>
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<tr>
<td>14</td>
<td>Urease test</td>
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<tr>
<td></td>
<td><strong>Test for Species Identification</strong></td>
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<td>16</td>
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<td>18</td>
<td>D-glucose, acid production</td>
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</tr>
<tr>
<td>19</td>
<td>D-glucose, gas production</td>
<td>Negative</td>
</tr>
</tbody>
</table>

PLATE 1. Growth of *Serratia marcescens* on Nutrient Agar medium

Optimization of pigment production

**Incubation period for the production of pigment from Serratia marcescens**

The optimum period for pigment production of *S. marcescens* was studied for 384 hours. Among this various incubation period, the results elucidated that 48 hours incubation showed the highest pigment production in *S. marcescens* and the results were represented in Figure 1.
Optimization of pigment production using different Broth

The production of pigment from *Serratia marcescens* were carried out using different broth such as Nutrient broth, Trypticase soya broth, Brain heart infusion broth, Peptone Glycerol broth and Modified broth. The study revealed that Trypticase soya broth showed highest pigment production in *Serratia marcescens*. Based on the intensity of the color production in trypticase soya broth the maximum pigment production was observed to be 0.37 were other are to be minimum. The results were similar to (Gerber 1979; Giri *et al*., 2004) shown in Figure 2.

Pigment production of *Serratia marcescens* in Trypticase soya Broth using different oil as substrate

The pigment production is characterized using trypticase soya broth with different oil substrates namely groundnut oil, coconut oil, olive oil, gingelly oil and castor oil. Among this depicts coconut oil showed maximum amount of pigment production in *S. marcescens* as given in Figure 3.
Figure 3. Effect of Trypticase soya broth with different oil as Substrate on *Serratia marcescens*

**Pigment production with different percentage of coconut oil**

The intensity of colored pigment production is analyzed using tripticase soya broth with different percentage of coconut oil as substrate ranges from 1 - 5%. The maximum pigment production was observed in 3% of coconut oil and the results were shown in Figure 4. (Sundaramoorthy *et al.*, 2009) reported the different oil substrates on pigment production.

Figure 4 .Effect of different percentage of coconut oil on *Serratia marcescens* for the production of pigment

**Optimization of Temperature for pigment production in *Serratia marcescens***

The effects of various temperatures such as 30°C, 35°C, 37°C, 45°C, and 48°C on *S. marcescens* for pigment production were studied. The maximum growth and color intensity were observed at 37°C. The results were shown in Figure 5. Sole *et al.*, (1994) studied using different temperature ranges.
Optimization of pH for pigment production in *Serratia marcescens*

The effects of different pH ranges from 5 - 9 were studied for the production of pigments. The results showed that highest pigment production were observed at pH 8 and were shown in Figure 6.

Purification of pigment by Column Chromatography

The extracted pigment was subjected to column chromatography for purification. 1ml fraction from the column was collected at a time interval of 1 hour. From the third to fifth fraction of the sample the pink color formation was appeared and their OD was similar to be 0.21. These fractions were pooled and taken for further studies. Plate 2 shows the formation of the pink color of the sample extract.
Extraction and Identification of pigment by Thin layer Chromatography

The eluted pigment fractions from the column chromatography were subjected to TLC silica gel plate to separate the pigments from the impurities. Pink color spots on the silica plate confirm the separation of the pigment.

CONCLUSION

Microorganisms have been used for a long time for production of molecules as antibiotics, enzymes, vitamins, texturing agents and so on. Microbial colors are used in various industries that enhance the commercial potential of the products. They are used in the production of various pigments like carotenoids, melanins, flavins, quinines, prodigiosins and more specifically monascins, violaecin or indigo. In the present study the pigment was isolated from the Gram-negative bacteria *serratia marcescens* which is characterized. The pigment was extracted and the sample was purified for further analysis. Purification of the pigment was done and separated. The pink color spot was observed under UV light which confirms the pigment as prodigiosin like compound. The properties of pigment can be further studied and can be used in drug development.

REFERENCES


