Prodigiosin production from *Serratia marcescens* strains obtained from farm soil

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ABSTRACT

Prodigiosin is a red color pigment produced by the bacterium *Serratia marcescens*. Prodigiosin is emerging as a valuable molecule because of its large applications. The pigment is anticancer, immunosuppressant, antifungal, algicidal and many more applications are still to be studied. It has already been proved that pigmented strain of *Serratia marcescens* is less virulent than non-pigmented strains. Moreover the strain we have obtained is from farm soil which indicates that prodigiosin production can be carried safely using this strain. In present study we employed an already characterized pigmented strain which was confirmed by morphological, biochemical and phylogenetic studies. A non-pigmented strain was used as negative control. The levels of prodigiosin are studied in peptone glycerol broth and nutrient broth. Influence of aeration is also studied which indicates that higher levels of prodigiosin are found in the media kept for aeration. We found that the prodigiosin production is increasing gradually after 48h and were maximum towards 72h thereafter the production was decreasing towards 92h in both media. Prodigiosin was nil after 24h also. Greater levels of prodigiosin were observed in nutrient broth which is quite significant. Generally oils are required for prodigiosin production but the strain is producing prodigiosin in nutrient broth which is indicative of wild type prodigiosin producing strain.

Keywords: *Serratia marcescens*, prodigiosin, periodic estimation, agitated, stationary.

1. Introduction

*Serratia marcescens* is a gram negative, non-motile, citrate positive bacterium. Colonies on nutrient agar are convex, circular with entire margin. Some strains produce characteristic red colour pigment called prodigiosin whereas rest does not. The red pigment prodigiosin was isolated from *S. marcescens* way back in 1902 by Kraft (Venil and Lakshmanperumalsamy, 2009). Prodigiosins are strong therapeutic molecules especially for their immunosuppressive properties and anticancer properties. The mechanism of action of these molecules is reviewed by Perez-Tomas et al., (2003). Four possible mechanisms are suggested attributed to prodigiosins as pH modulators, cell cycle inhibitors, DNA cleavage agents and mitogen activated protein kinase regulators. These molecules when combined with some other anticancer agents can greatly help in fighting cancer. The immunosuppressive properties were also carried earlier by Han et al., (1998). Many other applications are briefed as under. Effect of prodigiosin on human carcinoma cells was investigated by Kavitha et al., (2010) and significant results were found. Varied concentrations of prodigiosins were studied against percent decline in cancerous cells. Prodigiosin was also shown to be involved in apoptosis of haematopoietic cancer cell (Montaner et al., 2000). *Spodoptera litura* is one of the crop destructing insect. *Bacillus thuringiensis* toxin viz. Cry1C is the major weapon employed against these insects. Insecticidal activity was found to be enhanced when prodigiosin was
combined with Cry 1C. *S. marcescens* culture was grown in nutrient media and the pigment was obtained from the supernatant. The pigment was found to be 10% and was confirmed spectrophotometrically (Asano et al., 1999).

Algal bloom has been a threat to aquatic life with a slow but considerable damage. Aquatic life including fishes, prawns and aerobic bacteria are the major flora damaged. These also affect the ecosystem of the aquatic bodies as small as a pond to the seashore. Algicidal bacteria are one of the remedies to this damage Mayali and Azam (2004). The emergence of algicidal bacteria including prodigiosin is boon for aquatic life. Harmful algal blooms cause a massive economic loss and environmental disturbances. *Hahella chejuensis* is one of the prodigiosin producers to be employed as algicidal bacterium. This bacterium was initially undertaken for polysaccharide production but also showed red pigment production which was further chosen for algicidal studies with a very low concentration (1ppb) against *Cochlodinium polykrikoides*. The pigment from *Hahella chejuensis* was also studied as immunosuppressant and antitumor agent (Kim et al., 2008). Prodigiosins are found to selectively act on cancerous cells with a very less or no effect on normal cells. Drug resistant cells can particularly be targeted by prodigiosin. The mechanism of action is not clearly understood. It is also proposed that induction of DNA double breaks would be one mechanism and another being neutralization of pH gradient leading to apoptosis (Pandey et al., 2009). Parani and Saha (2008) optimized the medium for higher production of prodigiosin which included the casein-enriched medium supplemented with 4% vegetative oil mixture (sunflower, coconut and olive oil). Later antifungal activity was also studied against *Helminthosporium sativum*, *Fusarium oxysporium* and *Rhizoctonia solani*.

The pigment prodigiosin is also produced by *Streptomyces spectabilis* which was found to be antimalarial. The bacterium chosen was *Streptomyces spectabilis* producing metacycloprodigiosin with 50% activity against *Plasmodium falciparum* K1 without damaging normal cells (Isaka et al., 2002). These significant results can help in eradicating malarial parasites. Apart from prodigiosin production *S. marcescens* has many other remarkable applications. Species of *Serratia* are also helpful in enhancing the crop production by destructing a wide range of phytopathogenic fungi. The study was undertaken by Kamensky et al., (2003) against *Botrytis cinerea* gray mold and *Sclerotinia sclerotiorum* white mold diseases of cucumber which are quite popular. The antifungal activity was because of the chitinolytic and proteolytic activities of *Serratia plymuthica*. The strain was applied on the leaves which reduced the disease incidence by 76% and 80% respectively on *Botrytis cinerea* and *Sclerotinia sclerotiorum* respectively. The strain also produced the antibiotic pyrrolnitrin and siderophores as well as plant growth hormone indole-3-acetic acid. *Sclerotium rolfsi* is plant fungi. The biocontroll was studied using *Serratia marcescens*. The antifungal activity was because of chitinase production. The destruction of fungi up to 63% was achieved. *S. marcescens* was grown on components of *Sclerotium rolfsi*. N-acetyl- D-glucosamine was estimated as chitinolytic activity (Ordentlich et al., 1988).

Two chitinolytic enzymes were purified from *Serratia plymuthica* viz. endochitinase and one N-acetyl-beta-1,4- D-hexosaminidase. The enzyme acted optimally at temperature of 55 °C and pH of 5.4. Metals like Ca$^{2+}$, Co$^{2+}$ or Mn$^{2+}$enhanced the activity of the enzyme by 20%. An estimated 78 % inhibition of germination and 63.9 % inhibition of germ tube elongation was attained (Frankowski et.al. 2001). Two major enzymes endochitinase and a 98-kDa chitobiase were purified from *S. marcescens* strain by Someya et.al. (2001). The mold studied was *Botrytis cinerea* which was inhibited by these enzymes. However when prodigiosin was combined with these enzymes a synergistic effect was observed indicating multiple modes of actions. Such strategies can help in better biocontrol against various molds.

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Pyricularia oryzae is a major enemy of rice; a major crop consumed throughout India. Powder of S. marcescens was sprayed on rice plant. The bacterium was effective against Pyricularia oryzae. S. marcescens was isolated from various parts of the rice plants. The bio-control agent also survived for 80 days on rice plants (Jaiganesh et al., 2007). Regarding fertility aspects S. marcescens can solubilise phosphate. Phytic acid is converted into inorganic monophosphate making it available to plants. The prime criterion concerning enzyme preparation for various sources is resistance to temperature, higher stabilities and easy for handling (Mukesh Kumar et al., 2011).

Environmental applications of S. marcescens also have a great concern. Chromium has a higher toxicity levels. A strain of S. marcescens isolated from tannery effluent was found to remove 80% of tannery effluent. The strain could accumulate chromate on cell surface. Immobilization of S. marcescens can largely detoxify chromate (De Bruijn and Mondaca, 2000).

Enzyme productions have also been carried using S. marcescens. S. marcescens is also an efficient source of lipase enzymes. The enzyme acts optimally at pH 8.0 and 45 °C. The molecular weight of the enzyme was found to be 65 Da. The enzyme has marked stability in many water miscible and immiscible solvents (Zhao et al., 2008). Diltizem is a vasodilator used for various medical applications. The intermediates of these compounds are synthesized by using lipases obtained from S. marcescens (Hiroaki et al., 1996). An economic production of proteases from S. marcescens using whey as substrate is carried out by Romero et al., (2001). Two proteases metallo proteases and serine proteases were obtained with recovery of 15.7% and 9.9% respectively. Optimum pH and temperature for the enzyme activity was found to be 9.5 and 45°C.

As mentioned above species of S. marcescens have wide range of applications but the major being prodigiosin as anticancer agent. It is already stated that the pigmented strains are less virulent. Brazilian hospital was undertaken for studies on occurrence of S. marcescens where it was found that majority S. marcescens species (92%) belonged to non-pigmented species which were virulent to mice. A very low frequency of pigmented strain of S. marcescens was found to be virulent (Carbonell et al., 2000). This indicates that prodigiosin production can be carried out safely from S. marcescens. Moreover we have employed a strain isolated from farm soil. It is an already characterized strain. The production is carried out in nutrient broth and peptone glycerol broth, the frequently employed media for the production. The levels of prodigiosin are estimated periodically in agitated and stationary phases.

2. Materials and Methods

2.1 Prodigiosin production

A characterized strain of S. marcescens which was studied previously for DNase production was undertaken for prodigiosin production (Gene Bank Accession No. JF681182). A non-pigmented strain of S. marcescens was used as negative control (Gene Bank Accession No. JF681183). Phylogenetic studies, carbon sources utilization and morphological studies have been studied by Kamble (2011). Cultures were grown in nutrient broth and peptone glycerol broth, the mostly employed media for prodigiosin production. The production levels were estimated both at stationary and agitated phases to investigate the effect of aeration on production. The levels of prodigiosin in these conditions were estimated after 0hr, 24hr, 48hr and 72hr.
3. Extraction of prodigiosin

The cells were harvested by centrifugation at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in acidified ethanol (4% of 1M HCL in 96 ml ethanol). The mixture was vortexed and the suspension was centrifuged at 10,000 rpm for 10 min. The supernatant prodigiosin was transferred to the fresh vial (Slater et al., 2003).

4. Estimation of prodigiosin

The absorption pattern over various wavelengths was initially checked and it was found that the absorption maxima were at 499 nm where prodigiosin also absorbs maximally. At this wavelength the absorptions were recorded. The results were studied after above mentioned time intervals. The bacterial cell absorption prior to extraction was noted at every step. Isolated prodigiosin was estimated using the following formula (Mekhael and Yousif, 2009).

\[
\text{Prodigiosin unit/cell} = \frac{[\text{OD}_{499} - (1.381 \times \text{OD}_{620})] \times 1000}{\text{OD}_{620}}
\]

OD- Optical density; \(\text{OD}_{499}\) – Pigment absorbance ; \(\text{OD}_{620}\) – Bacterial cell absorbance

1.381 – Constant

5. Results and discussion

Culture of \(S. \) marcescens was inoculated on above mentioned media. Prodigiosin production can be visualized in culture flask (Fig1 and 2). Optical densities of the extracted prodigiosin Prodigiosin production was estimated periodically after intervals of 24 hr, 48 hr and 72 hr. Prodigiosin production was nil after 24 hr in stationary phase determined spectrophotometrically at 499 nm. (Mekhael and Yousif, 2009). Prodigiosin unit per cell after 48 hr was found to be 1070.33 and 1188.94 in nutrient broth and peptone glycerol broth respectively. Maximum production was reported in 72 hr thereafter production ceased towards 96 hr. In stationary phase the prodigiosin unit per cell in nutrient broth and peptone glycerol broth was found to be 1246.1 and 1055.96. The lowest prodigiosin unit per cell was found after 96 hr i.e. in nutrient broth it was 957.1 and in peptone glycerol broth i.e. 885.38 (Fig3 and 4).

Table 1: Pigment Absorbance at 499 nm

<table>
<thead>
<tr>
<th>Hours</th>
<th>Nutrient Broth</th>
<th>Peptone Glycerol Broth</th>
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<tbody>
<tr>
<td></td>
<td>Agitated</td>
<td>Stationary</td>
</tr>
<tr>
<td>24h</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>48h</td>
<td>1.716</td>
<td>1.673</td>
</tr>
<tr>
<td>72h</td>
<td>1.980</td>
<td>1.686</td>
</tr>
<tr>
<td>96h</td>
<td>1.692</td>
<td>1.580</td>
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</table>
Table 2: Total prodigiosin estimated in nutrient broth and peptone glycerol broth in stationary and agitated phase

<table>
<thead>
<tr>
<th>Hours</th>
<th>Total prodigiosin in N.B mg/lit</th>
<th>Total prodigiosin in P.G mg/lit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Agitated</td>
<td>Stationary</td>
</tr>
<tr>
<td>24h</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>48h</td>
<td>1335.44</td>
<td>1188.94</td>
</tr>
<tr>
<td>72h</td>
<td>1845.27</td>
<td>1246.10</td>
</tr>
<tr>
<td>96h</td>
<td>1301.10</td>
<td>957.10</td>
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</table>

Figure 1 and 2: Broth cultures of non-pigmented and pigmented strain of S. marcescens (Right). Broth cultures with highest levels of prodigiosin (Left)

Figure 3 and 4: Prodigiosin extraction from peptone glycerol broth and nutrient broth in agitated phase
Prodigiosin production in nutrient broth and peptone glycerol broth with shaking after 24h was nil. Sufficient production was observed after 48h i.e. 1335.44 and 1238.23 in nutrient broth and peptone glycerol broth. Maximum in both the media was observed in 72h i.e. 1845.27 and 1335.38 in nutrient broth and peptone glycerol broth. Production was seen to decrease in 92h; it was 1301.10 and 1271.21 in nutrient broth and peptone glycerol broth respectively. Study shows that aeration resulted in enhanced levels of prodigiosin. The result indicated that the maximum amount of prodigiosin is produced in nutrient broth with shaking in comparison with peptone glycerol broth. Oils are used for greater productivity of prodigiosin but the strain we employed was found to produce more levels of prodigiosin in nutrient broth which is an indicative of wild type strain.

6. References


