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Research Article

OPTIMIZATION AND PRODUCTION OF PRODIGIOSIN FROM SERRATIA MARCESCENS MBB05 USING VARIOUS NATURAL SUBSTRATES

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ABSTRACT

Prodigiosin produced by *Serratia marcescens* is a promising drug owing to its reported characteristics of having antibacterial, antimalarial, antimycotic, immunomodulating and antitumor activities. Investigations were made in the present study to screen new strain of *Serratia marcescens* MBB05; the natural red pigment prodigiosin producing strain was isolated from Western Ghat Ecosystem, Tamil Nadu, India. The regular liquid media currently being used for prodigiosin biosynthesis are nutrient broth, peptone glycerol broth and production medium. For this, eleven natural substrates have been tested. Among these substrates, peanut powder was found to be the best natural substrate at a concentration of 2.0% in distilled water at pH-7; inoculum (5%); temperature (30°C); incubation period of 36 hours. The production was 4.5 times higher (560.4 mg/mL) than the optimized basal medium. A natural media which supports the growth of the bacteria and at the same time prove efficient to activate high levels of pigment production was the aim of this work.

Keywords: prodigiosin; *Serratia marcescens*; natural substrate; peanut powder.

INTRODUCTION

Prodigiosins, a family of natural red pigments characterized by a common pyrrolylpyrromethane skeleton, are produced by various bacteria that first characterized from *Serratia marcescens*. Bacteria possess huge ability in producing biopigments that are synthesized for producing medicinally important products[1]. These pigments are emerging as a novel group of compounds having distinct biological activities antibacterial, antimalarial, antimycotic, immunomodulating, antitumor and nuclease[2, 3, 4]. Synthesize large quantities of the pigment a comparative study of different media, role of temperature, growth of the organism in the different media and pigment production must be studied[5, 6]. Prodigiosins are a family of naturally occurring tripyrrole ring-containing red pigments having a common pyrrolyldipyrrolylmethene skeleton. Prodigiosin, $C_{20}H_{25}N_3O$, has an unusual structure with three pyrrole rings and is a pyrryldipyrrylmethane; two of the rings are directly linked to each other, and the third is attached by way of a methane bridge. It forms lustrous, square pyramidal crystals that are dark red with a green reflex; the hydrochloride[7, 8, 9]. Prodigiosin has been shown to be active against multidrug resistant cancer cell lines. It has been reported that prodigiosin members show anticancer properties through induction of apoptosis by activating either or both of the p38-MAPK and SAPK/JNK pathways[10]. In recent years, SSF has shown much promise in the development of bioprocesses and products. More recently, it has gained importance in the production of microbial enzymes due to several economic advantages over conventional submerged fermentation[11]. Our aim is to find out a media that may support the growth of the bacteria and at the same time prove efficient to trigger high levels of pigment production. The present study we have selected eleven natural substrates namely black sesame powder, coconut oil, coconut powder, fenugreek powder, mustard oil, mustard powder, olive oil, peanut oil, peanut powder, sesame oil and white sesame powder, have been planned to test their efficiency in fulfilling our aim.

MATERIALS AND METHODS

Isolation and screening of bacteria for prodigiosin production

Serratia marcescens MBB05 was isolated from soil samples collected from Western Ghat Ecosystem forest, Coimbatore, Tamil Nadu, India, in a sterile sample container for the isolation of pigment producing bacteria. Samples were air-dried at 30°C for 2-6 days and stored in a sealed plastic container before use. 1 g of soil sample was serially diluted (10^{-2} to 10^{-7}) with sterile distilled water and spread plated on nutrient agar (g/L). Four potential isolates were isolated and it was used for prodigiosin production. The culture was maintained on nutrient agar slants and stored at 4°C until further use. For screening, hundred mL conical flasks containing 50mL of nutrient broth (g/L) was prepared and used. 48 hours old inoculum (5%) was added to each of the flasks and incubated at 27°C in a sterile condition. After 96 h of incubation period broth was taken for pigment extraction and extra cellular protein estimation. After 96 h of incubation the 1 mL medium was centrifuged at 10,000 x *g* for 10 min at 4°C. Pigment was extracted from cell pellets by shaking in 1 mL of acidic methanol (96 mL methanol and 4 mL HCl) for 35 min at 30°C. Debris was removed by centrifugation at 10,000 x *g* for 20 min at 4°C. The methanolic extract of the pigment was evaporated at room temperature and it was dissolved in approximately 3 mL of chloroform and transferred into fresh sterile micro tubes. It was again evaporated at room temperature to concentrate the pigment[12].

Estimation of prodigiosin

Prodigiosin yield was measured from a calibration curve based on the A535 of standard prodigiosin[12]. The acidified prodigiosin was measured at 535 nm using spectrophotometer.

Isolation of chromosomal DNA and Molecular identification

The template DNA (selected bacterial strains) for the PCR amplification was isolated by modification of the methods suggested by Cook and Meyers[13] and that of Coombs and Franco[14]. The potential isolate was further confirmed at the species level based on the 16s rRNA sequence alignment. PCR amplification (Weisburg *et al.*[15]) of the 16S rRNA gene was performed using two universal oligonucleotide bacterial primers, 16S rRNA forward primer: *AGA GTT TGA TCC TGG CTC AG,* 16S rRNA reverse primer: *ACG GCT ACC TTG TTA CGA CTT*. The 16S rRNA gene sequence of the potential isolate was compared with available 16S rRNA gene sequences in GenBank databases using the BLAST search facility at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Optimization studies

Effect of various media on prodigiosin production

The hundred mL conical flasks containing 50 mL of nutrient broth (g/L) was prepared and sterilized separately. 48 h old inoculum (5%) was added to each of the flasks and incubated at 27°C in a sterile condition. After 48 h of incubation period broth was taken for pigment extraction, estimation as stated above.

Effect of various physico-chemical parameters on prodigiosin production

To determine the increase the potentiality of the bacteria to synthesize large quantities of the pigment a comparative study of different media with physico-chemical parameters were studied. The components of the different media were analysed and compared effectively, to deduce the most probable reason for the increase or the turn down in pigment production.

Effect of temperature

The pigment production was carried out at 27, 28, 30, 32 and 37°C keeping all other conditions at their standard levels and then assayed for prodigiosin. Cultivation temperatures on prodigiosin production have been carried out by incubating the nutrient broth at different temperatures. The optimum temperature achieved by this step was fixed for subsequent experiments.

Influence of incubation time

In order to the optimum incubation period for prodigiosin production, nutrient broth were incubated for different time durations (12, 24, 36, 48, 60, 72, 84 and 96 h) and then assayed for prodigiosin. The other conditions were 5% of inoculum level at pH 7 and the incubation was carried out at 30°C. The optimum incubation period achieved by this step was fixed for subsequent experiments.

Effect of inoculum size

To evaluate the effect of inoculum size on prodigiosin production varied cell concentrations (2.5, 5.0 and 7.5%) were added to different flasks containing nutrient broth and then assayed for prodigiosin production. The fermentation was carried out at 30°C keeping all other conditions at their optimum levels. The optimum inoculum level achieved by this step was fixed for subsequent experiments.

Effect of pH

To determine the effect of pH of the nutrient broth on prodigiosin production, experiments were performed with media of different pH. While optimizing the pH of the basal medium, the pH of aqueous solution was varied from 5.0 to 9.0 with 0.1 M NaOH or 0.1 N HCl and then assayed for prodigiosin. The optimum pH achieved by this step was fixed for subsequent experiments.

Role of different carbon sources

To evaluate the effect of different carbon sources such as arabinose, ethanol, fructose, galactose, glucose, glycerol, lactose, maltose, and sucrose were supplemented separately to a final concentration of 0.5% (w/v) in the nutrient broth. After incubation in an optimal condition the prodigiosin was quantified.

Role of different nitrogen sources

In order to study the effect of different nitrogen sources such as ammonium chloride, ammonium nitrate, ammonium sulphate and dried yeast were supplemented separately to a final concentration of 0.5% (w/v) in nutrient broth. After incubation in an optimal condition the prodigiosin was studied.

Influence of different amino acids

To determine the effect of different amino acids such as cysteine, leucine, methionine, tryptophan and proline (0.5 %, final concentration) were dissolved in the nutrient broth. After incubation in an optimal condition the prodigiosin was estimated.

Effect of various concentrations of glucose, dried yeast and cysteine on prodigiosin

Production

Investigations on the various concentrations of glucose, dried yeast and cysteine (0.25, 0.5 and 0.75 %) was added to the sterile nutrient broth separately. 48 h old inoculum (5%) was added to each of the flasks and incubated at 30°C in a sterile condition. After 36 h of incubation period broth was taken for pigment extraction and estimation.

Effect of various natural substrates

To evaluate the effect of different natural substrates, coconut oil, mustard oil, olive oil, peanut oil, sesame oil, black sesame powder, coconut powder, fenugreek powder, mustard powder, peanut powder and white sesame powder were supplemented to a concentration of 2% in distilled water. After incubation in an optimal condition the prodigiosin was quantified.

Screening of various media on production of prodigiosin using peanut powder

The hundred mL conical flasks containing 50 mL of nutrient broth (g/L) (peanut powder: 2; peptone: 5; beef extract: 3; yeast extract: 2; sodium chloride: 5; pH: 7.0 ± 0.2), peptone glycerol broth (g/L) (peanut powder: 2; meat extract 10; peptone 10; glycerol 10; pH: 7.0 ± 0.2), and peanut powder (2%) in distilled water (pH: 7.0 ± 0.2) was prepared and sterilized separately. 48 h old inoculum (5%) was added to each of the flasks and incubated at 30°C in a sterile condition. After 36 h of incubation period broth was taken for pigment extraction and estimation.

Effect of various concentration of peanut powder on prodigiosin production

The hundred mL conical flasks containing 50 mL of peanut powder (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0%) in distilled water was prepared and sterilized separately. 48 h old inoculum (5%) was added to each of the flasks and incubated at 30°C in a sterile condition. After 36 h of incubation period broth was taken for pigment extraction and estimation.

RESULTS AND DISCUSSION

Isolation and screening of *Serratia marcescens* MBB05 for prodigiosin production

Ten soil samples were collected from different parts of Western Ghat Ecosystem forest and screened for prodigiosin producing organism. Among the 10 soil samples red pigmented colonies were isolated from 5 soil samples. The amount of prodigiosin produced by the 4 bacterial isolates was estimated. Among the four bacterial isolates tested for prodigiosin production, two organisms were able to produce pigment ranging from 10 to 165 mg/mL. Highest production was noticed in isolate *Serratia marcescens* MBB05 with 123 mg/mL of prodigiosin and 168 mg/mL of protein. Based on their highest prodigiosin production the bacterial strain *Serratia marcescens* MBB05 was selected for further studies. Among 105 *Serratia* spp. isolated from clinical and environmental sources, 28 produced prodigiosin reported as Gargallo-viola *et al*. [16]. The present study, four bacterial strains were isolated from western ghat ecosystem forest soil area, MBB05 bacterial strain produced prodigiosin. An ethanol utilizing *Serratia marcescens* S389 from the soil sample, produced prodigiosin up to 3 mg/mL has also isolated[12].

Molecular identification

PCR amplification and sequencing of the 16SrRNA genes

The Potential isolate was further confirmed till the species level by using primers specifically designed for the identification of *Serratia marcescens* MBB05 based on the 16s rRNA sequence alignment of soil isolates. The partial nucleotide sequence for the 16S rRNA gene (581 bp) reported, which showed more than 99% similarity to *S. marcescens.* The submitted sequence appears in the GenBank nucleotide sequences databases under accession number GU186412 for *S. marcescens* MBB05. The level of DNA–DNA hybridization between strain *S. marcescens* KREDT and *S. marcescens* JCM 1239T was found to be 97% similarity which was reported by Ajithkumar *et al.*[17]. A strain that was found to be 88% similar to *S. marcescens* (AJ233431). The profile of the selected strain was also similar to *S. marcescens* isolated by Deorukhkar *et al.*[18].

Production of prodigiosin

Effect of different media on prodigiosin production

Among the two media were tested for prodigiosin production, *viz.,* nutrient broth and peptone glycerol broth. *S. marcescens* MBB05 showed prodigiosin production 135.5 mg/mL compared to 101 mg/mL in peptone glycerol broth. Extra cellular protein production

of nutrient broth showed 44.2 mg/mL and 98.4 mg/mL noticed in peptone glycerol broth. Comparing the results, nutrient broth showed better prodigiosin production. Hence, the nutrient broth has been selected for further optimization studies. Anuradha *et al.*[19] has reported that the production of prodigiosin was more in nutrient broth (0.52 mg/mL) than peptone glycerol broth (0.302 mg/mL).

In our study the potential strains has produced 112.1 – 166.3 mg/mL, which was much higher reported earlier. Both in nutrient broth and peptone glycerol broth the major components were peptone, meat and yeast extract. Peptone is a commercially available digest of a particular plant or animal protein, made available to organisms as peptides and amino acids to help satisfy requirements for nitrogen, sulfur, carbon and energy. Yeast and meat extracts contain eukaryotic tissues (yeast, beef muscle, liver, brain, heart, etc.) that are extracted by boiling and then concentrated to a paste or dried to a powder. These extracts are frequently used as a source of amino acids, vitamins and coenzymes, growth factors by fastidious organisms. In peptone glycerol broth, the glycerol was the carbon source. Anuradha *et al.*[19] has reported that the seeds and oils contain metals; vitamins, saturated and unsaturated fatty acids and the concentration of these components are variable in each kind of seed or oil.

Effect of various physico-chemical parameters for prodigiosin production

Production of prodigiosin is greatly influenced by physical factors such as temperature, pH, incubation time, inoculum, substrate concentration and media components, especially carbon and nitrogen sources. It is important to find out an inexpensive and optimized media for the production of prodigiosin. So a study on the influence of various physico-chemical parameters were necessary. Initially one parameter was evaluated and it was then incorporated at its optimized level in the subsequent experiments using nutrient broth medium**.**

Effect of temperature

In this optimization study, 27, 28, 30, 32 and 37°C were the temperature selected. There was less prodigiosin production when the nutrient broth were 131.5, 143.2, 129.4, 15.3 mg/mL (*S. marcescens* MBB05), incubated at 27, 28, 32 and 37°C respectively. The optimum temperature of incubation for the selected bacterial strain was 30°C. The prodigiosin production was 151.0 mg/mL by *S. marcescens* MBB05 (Fig.1).

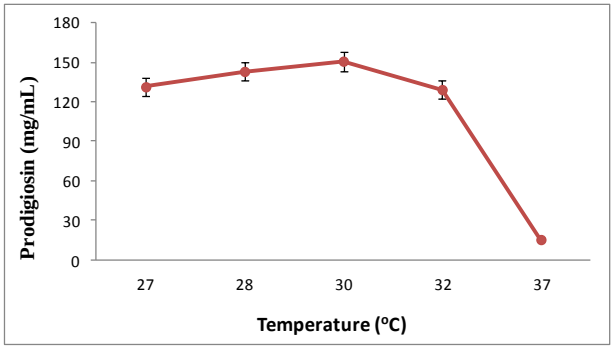

Fig.1:Effect of temperature on production of prodigiosin by *S. marcescens* MBB05 (Nutrient broth; inoculum 5%; temperature 30°C; incubation period 36 h; results are mean of independent experiments ± SD and are expressed as mg/mL

The maximum yield of prodigiosin was observed at 28°C for nutrient broth and sesame broth, but in peptone glycerol broth maximum pigment production was at 30°C, reported by Anuradha *et al.*[19]. They also reported that a block in prodigiosin production above 30°C in nutrient broth, and at 37°C *Serratia marcescens* did not show any pigment production in nutrient broth and the culture broth was white in color. Nutrient broth and peptone glycerol broth the prodigiosin production was completely blocked at 37°C which was reported by Pryce and Terry, [20]. These reports are in agreement with the present finding. This might be due to the fact that the terminal step in prodigiosin biosynthesis i.e., condensing of mono and bipyrrole moieties was temperature sensitive[21]. The maximal amounts of prodigiosin were synthesized in either minimal or completed medium after incubation of cultures at 27°C for 7 days which was reported by Williams *et al.*[22].

Effect of incubation time

The optimization of the prodigiosin production parameter was initiated by incubating the selected strains at different time durations of 12 h equal intervals. The selected isolate prodigiosin production ranged between 32.2 and 116.1 mg/mL during 60, 72, 84 and 96 h of incubation, and whereas at 12, 24, 36 and 48 h it ranges between 110 to 177 mg/mL.

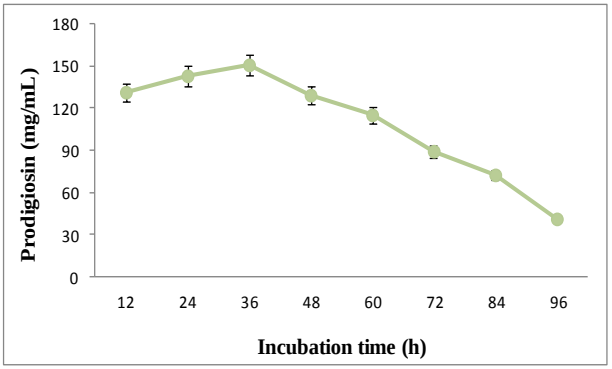

Fig.2:Effect of Incubation time on production of prodigiosin by *S. marcescens* MBB05 (Nutrient broth; inoculum 5%; temperature 30°C; incubation period 36 h; results are mean of independent experiments ± SD and are expressed as mg/mL.

The production of prodigiosin pigment reached 151 mg/mL on the 36 h of incubation, which was the maximum compared to all other incubation timings (Fig.2). When cultures of *Serratia marcescens* were incubated at 27°C in 1% casein hydrolysate, viable count and protein attained maximal values within 24 to 48 h, where as prodigiosin did not reach a maximum until 96 h which was reported by Williams *et al.*[22]. Cang *et al.*[12] has reported that the maximum prodigiosin production was noticed at 48 h and that the production has completed by 72 h.

Effect of inoculum size

An investigation for further increase in prodigiosin production, the effect of various inoculum size were studied. To evaluate the effect of inoculum size, varied cell concentrations (2.5, 5.0 and 7.5%) were added to different flasks and then carried out as described in materials and methods. Prodigiosin production varied with inoculum level and showed parabolic nature in the studied range (Fig.3). The maximum prodigiosin production 149.2 mg/mL was observed at 5.0% for *S. marcescens* MBB05. Prodigiosin production was comparatively less at 2.5 and 7.5% inoculum concentration.

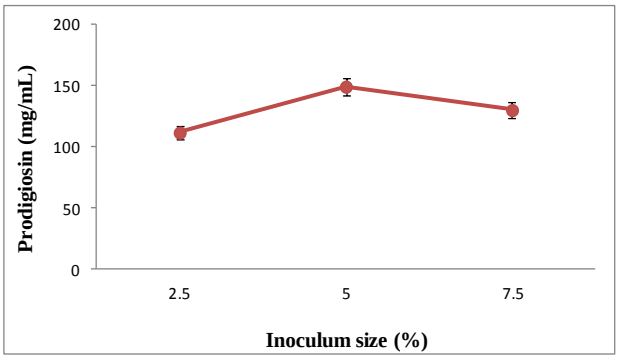

Fig.3. Effect of Inoculum size (%) on production of prodigiosin by *S. marcescens* MBB05 (Nutrient broth; inoculum 5%; temperature 30°C; incubation period 36 h; results are mean of independent experiments ± SD and are expressed as mg/mL.

Effect of pH

The selected potential bacterial strains were subjected to various pH ranging from 5.0 to 9.0 were taken for the study with an interval of pH 0.5. Less prodigiosin production were noticed in the acidic (5.0 to 6.0) and alkaline (8.0 to 9.5) condition with 54.1 as the least and 164.4 mg/mL as its highest. But at the neutral (6.5 to 7.5) range the production was 129.9 to 171.1 mg/mL.

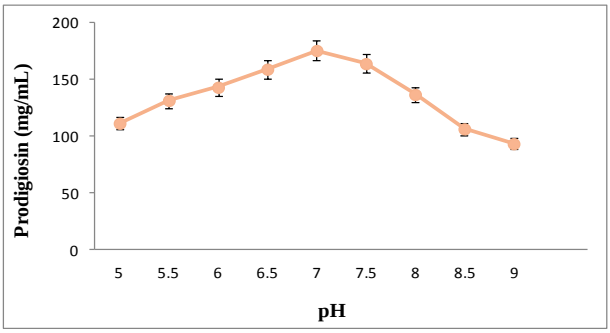

Fig.4:Effect of pH on production of prodigiosin by *S. marcescens* MBB05 (Nutrient broth; inoculum 5%; temperature 30°C; incubation period 36 h; results are mean of independent experiments ± SD and are expressed as mg/mL.

Investigation on this study, *S. marcescens* MBB05 has produced 175.6 mg/mL which was the highest production recorded in the pH 7.0 (Fig.4). Hence pH 7 was maintained in optimization studies. The influence of external pH on the regulation of biosynthesis of secondary metabolites has been described previously[23, 24, 25, 26]. In some cases, the optimum for biomass yield was different from that for metabolite production[24, 25]. *Serratia marcescens* was considered to grow optimally at pH 6.4-7.4. Sole *et al.*[27] has reported that the maximum pigment production was detected at pH 8.4-8.5, and he demonstrated that, at pH values ranging from 5.5 to 9.5 the pigment production was pH-dependent and the optimum pH was in the range 8.0-8.5. The initial pH of the media affected the biosynthesis of prodigiosin by growing cultures in glucose media: if the initial pH was 5.0, no prodigiosin was produced, but pH values up to 8 had no effect which was reported by Woods *et al.*[28]. However, cultures of *S. marcescens* have a powerful buffering capacity. The irrespective of initial pH of media, the final pH was 7.2 to 8.0 as the bacteria grow which has been reported by Ruis *et al.*[29] and Williams and Qadri[30]. In our present study, the maximum prodigiosin was noticed in the medium at the pH of 7.0.

Role of different carbon sources

In order to study the effect of different carbon sources on prodigiosin pigment production, 0.5% of arabinose, ethanol, fructose, galactose, glucose, glycerol, lactose, maltose, and sucrose were separately added to the test medium inoculated with the selected bacterial strains as per the method stated in the previous chapter. Effect of ethanol, glucose and glycerol as a carbon source has greatly influenced the selected strain for the prodigiosin production. Prodigiosin production was 183.4 mg/mL for ethanol, 188.4 mg/mL for glucose and 179.6 mg/mL for glycerol inoculated with *S. marcescens* MBB05 (Fig.5). Less production was noticed in case of lactose supplemented medium. Since *Serratia* sp. are non lactose fermenter the growth and other parameters would have reduce the pigment production. Arabinose, fructose, galactose, maltose and sucrose were moderately influencing the prodigiosin production. In the bioreactor study with an internal adsorbent for prodigiosin the final yield was 13 mg/mL[31] and the media used had dextrose in the culture broth and casein in production medium. A medium containing ethanol as the carbon source the yield was 3 mg/mL which was reported by Cang *et al.*[12]. The reduction in prodigiosin production by *Serratia marcescens* mediated by glucose and other metabolizable sugar was due to a decrease in pH observed in the cell suspensions[1]. Tao *et al.*[32] has reported that the prodigiosin production was more in glycerol followed by maltose. During the course of screening for ethanol-utilizing bacteria capable of producing a bioactive metabolite from ethanol Cang *et al.*[12] has found that several bacterial isolates produced an antimicrobial agent only when grown on ethanol. A high yield of pyoluteorin and 2, 4-diacetylphloroglucinol by one such isolates of *Pseudomonas fluorescens* S272, from ethanol has been reported by Yuan *et al.*[33]. The isolate *Serratia marcescens* S389 produced about 3 mg/mL of prodigiosin when grown on ethanol under the appropriate conditions which was also reported by Cang *et al.*[12]. In the present study the maximum prodigiosin was noticed in the medium containing glucose as the substrate.

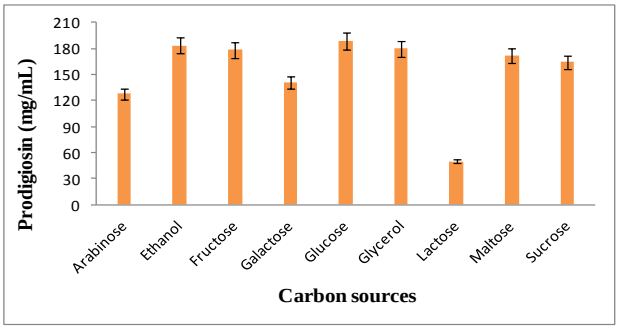

Fig.5: Effect of Carbon sources on production of prodigiosin by *S. marcescens* MBB05 (Nutrient broth; inoculum 5%; temperature 30°C; incubation period 36 h; results are mean of independent experiments ± SD and are expressed as mg/mL.

Effect of various concentrations of glucose

Glucose concentration of 0.5 % was optimum for the production of prodigiosin. *S. marcescens* MBB05 has produced 222.4 mg/mL of prodigiosin at 0.5 % concentration of glucose (Fig.6). At 0.25 and 0.75 % concentration of glucose the production was 196.4, 108.8 mg/mL respectively. Glucose, usually an excellent carbon source for growth, interferes with the synthesis of many secondary metabolites. Because of parallels with the well-known suppression by glucose of catabolic enzymes that use less-preferred substrate, this has been referred to as 'catabolite repression'. In many secondary metabolite pathways, the enzymes subject to control by the carbon source are known [34, 35]. Prodigiosin production is inhibited when glucose is added to the growth medium [10, 36]. This substrate inhibits the synthesis of the pigment in cultures grown on solid medium with concentrations up to 15g/L, and there was a close correlation between glucose consumption and the synthesis of this secondary metabolite[36]. In some micro-organisms, carbon catabolite repression of enzymes that are essential for nutrient utilization is associated with the transcriptional control that involves cyclic adenosine 3, 5-monophosphate (cAMP) as a positive effector[37, 38]. The metabolic role of cAMP in prokaryotes is not limited to controlling transcription of catabolic enzymes but is also required for other functions not directly related to catabolism[39]. However, cAMP does not appear to be involved in the glucose effect, on the synthesis of some secondary metabolites[40, 41, 42].

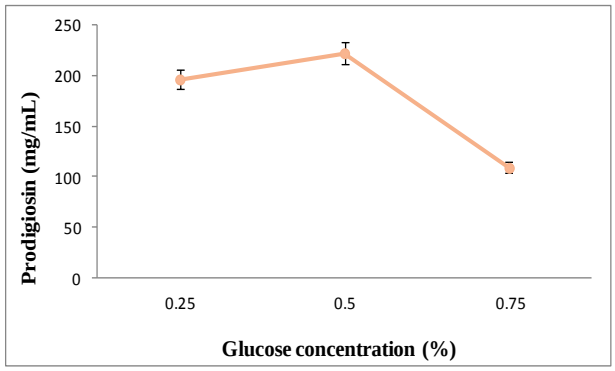

Fig.6. Effect of various glucose concentration on production of prodigiosin by *S. marcescens* MBB05 (Nutrient broth; inoculum 5%; temperature 30°C; incubation period 36 h;

results are mean of independent experiments ± SD and are expressed as mg/mL.

Role of different nitrogen sources

Different nitrogen sources *viz.,* ammonium chloride, ammonium nitrate, ammonium sulphate and dried yeast were tested. Nitrogen source is essential for the organism for prodigiosin production. Dried yeast supported all the three selected isolates for maximum prodigiosin pigment production. Strain *S. marcescens* MBB05 produced 186.8 mg/mL mg/mL of prodigiosin. 84.4, 103.4 and 79.3 mg/mL prodigiosin was produced when ammonium chloride, ammonium nitrate and ammonium sulphate was used respectively (Fig.7). The various nitrogen sources studied by Cang *et al.*[12] for the prodigiosin production, pharmamedia and polypepton gave a good antibiotic production as well as good bacterial growth and they also states that pharmamedia gave the best yield. The present study revealed that the prodigiosin production was very low when incorporated with various inorganic phosphates. This may be due to that, the production of various secondary metabolites in some Gram-negative bacteria under the *N*-acylhomoserine lactore–regulatory mechanism is known to respond to phosphate starvation[43], it seems that the promotion of prodigiosin production by strain *Serratia marcescens* S389 under phosphate limitation is reasonable. Good production was noticed in the case of dried yeast supplemented medium. This may be due to containing of amino acids, vitamins and coenzyme to promote the production of prodigiosin.

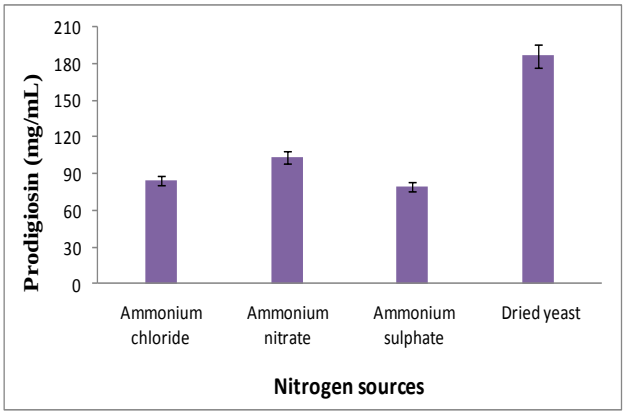

Fig.7: Effect of Nitrogen sources on production of prodigiosin by *S. marcescens* MBB05 (Nutrient broth; inoculum 5%; temperature 30°C; incubation period 36 h; results are mean of independent experiments ± SD and are expressed as mg/mL.

Effect of various concentrations of dried yeast extract

Yeast extract concentration of 0.5% was optimum for the production of prodigiosin. *S. marcescens* MBB05 has produced 181.7 mg/mL of prodigiosin at 0.5 % concentration of yeast extract (Fig.8).

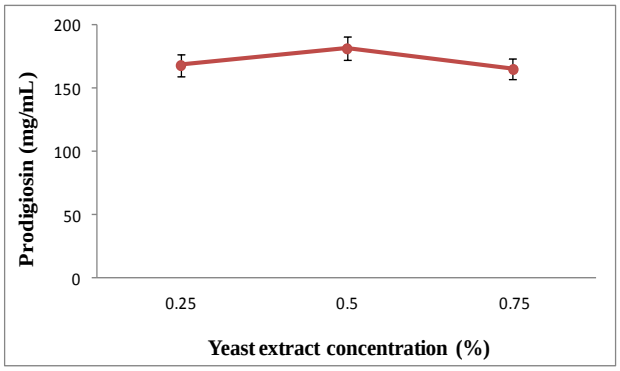

Fig.8: Effect of various yeast concentration on production of prodigiosin by *S. marcescens* MBB05 (Nutrient broth; inoculum 5%; temperature 30°C; incubation period 36 h; results are mean of independent experiments ± SD and are expressed as mg/mL.

Effect of different amino acids

In order to study the effect of different amino acids sources, proline, methionine, tryptophan, leucine and cysteine were tested. Cysteine containing nutrient broth media supported for maximum prodigiosin pigment production 192.2 mg/mL (Fig.9). The next best amino acid was proline that produced 181.6 mg/mL. When tryptophan, leucine and methionine were added to the nutrient broth the production ranges from 101.2 to 172.9 mg/mL of prodigiosin. Based on this study, cysteine was the best amino acid that was selected for production of prodigiosin. The proline did not cause biosynthesis of prodigiosin in non-proliferating cells unless it was catabolized which was demonstrated by Scott *et al.*[44].

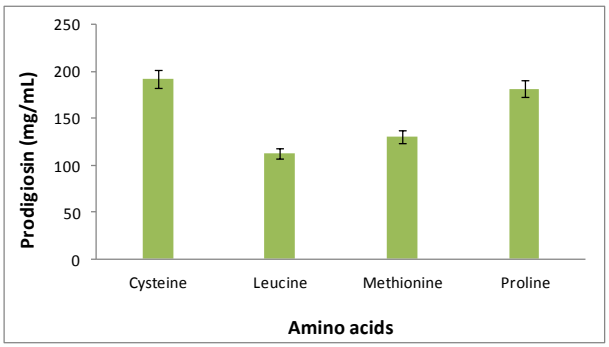

Fig.9:Effect of Amino acids on production of prodigiosin by *S. marcescens* MBB05 (Nutrient broth; inoculum 5%; temperature 30°C; incubation period 36 h; results are mean of independent experiments ± SD and are expressed as mg/mL.

Proline oxidase is the first enzyme in proline degradation and its activity rises during the first 8 h of incubation. Mutants incapable of utilizing proline as either carbon or nitrogen sources for growth did not form pigment in NPC suspensions during incubation with L-proline. Only amino acids that were utilized as sources of both carbon and nitrogen for growth resulted in biosynthesis of prodigiosin in nonproliferating cells of *S. marcescens*[45]. After addition of the amino acids, and before the appearance of prodigiosin, the rates of synthesis of DNA, RNA, and protein increased. The significant metabolism occurred in the bacteria and that the added amino acids were used for other cellular activities, probably as sources of energy, of intermediates, and for synthesis of enzymes, in addition to biosynthesis of prodigiosin. Such utilization of amino acids probably accounted for the high concentrations of effective amino acids required for synthesis of prodigiosin by non-proliferating cells[46, 49].

In the present work, the cysteine was promoted high prodigiosin production when compared to other amino acid supplementation in the medium. These amino acids probably supplied major pools of intermediates for metabolic processes. Amino acids that effected biosynthesis of prodigiosin in non-proliferating cells may serve a dual role in biosynthesis of prodigiosin. They can be sources of carbon and nitrogen for cellular metabolism, and they can be direct precursors of the pigment. Proline seems to serve a dual role[45]. Tanaka *et al.*[47] and Wasserman *et al.*[48] have reported that the ring of proline entered intact into the bipyrrole part of the prodigiosin molecule.

Effect of various concentrations of cysteine

Cysteine concentration of 0.5% was optimum for the production of prodigiosin. *S. marcescens* MBB05 has produced 207.2 mg/mL of prodigiosin at 0.5 % concentration of cysteine (Fig.10).

Effect of various natural substrates

For the selection of suitable substrates, eleven natural substrates have been tested namely black sesame powder, coconut oil, coconut powder, fenugreek powder, mustard oil, mustard powder, olive oil, peanut oil, peanut powder, sesame oil and white sesame powder. *S. marcescens* MBB05, the highest prodigiosin production was seen in peanut powder as substrate, with the production of 466.5 mg/mL

respectively (Fig.11). It is also noted that olive oil ranked low in prodigiosin production, 135.1 mg/mL (*S. marcescens* MBB05). MBB05 has produced prodigiosin in the order of substrate suitability, which was peanut powder > peanut oil > black sesame powder > fenugreek powder > mustard oil > mustard powder > white sesame powder > sesame oil > coconut oil > coconut powder > olive oil. The oils are known for their high levels of unsaturated fatty acid content and a very low percentage of saturated fatty acids which was studied and reported by Anuradha *et al.*[19]. From the results observed by them the pigment yield was 15 times more in media containing fatty acid seeds than in oils. The oil gave a better yield over the various carbons (not fatty acid containing seeds) and nitrogen sources tested[49]. The oil has given a better yield when compared to nutrient broth and peptone glycerol broth. Even this low level could be due to the presence of low concentration of saturated fatty acid present in oils[19]. They also reported that the prodigiosin yield was higher in peanut oil broth when compared to sesame oil broth, but the level of unsaturated fatty acid is higher (~47%) in sesame oil. They also proposed that the bonded fatty acids as carbon source were less accessible by *Serratia marcescens*. According to Nakamura[50] in his patent describes that the use of sodium oleate media and the substitution of sodium oleate with oleic acid and has used only triolein as substrate and reported 0.69 mg/mL yield of pigment. The bonded fatty acids as carbon source are less accessible by Serratia marcescens[51]. In the present study, peanut powder gave excellent substrate for prodigiosin production. In this context, the substrate peanut powder may contain necessary carbon, nitrogen and essential micro nutrients for higher production of prodigiosin.

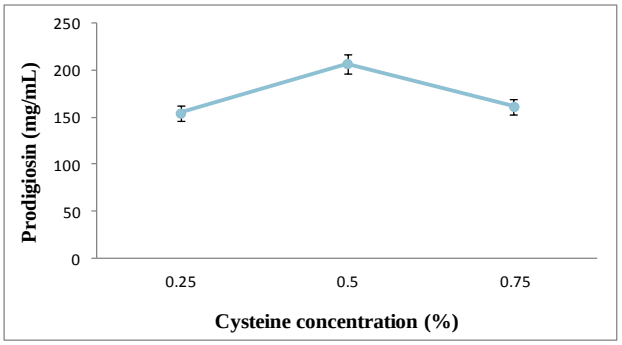

Fig.10:Effect of various cysteine concentration on production of prodigiosin by *S. marcescens* MBB05 (Nutrient broth; inoculum 5%; temperature 30°C; incubation period 36 h; results are mean of independent experiments ± SD and are expressed as mg/mL.

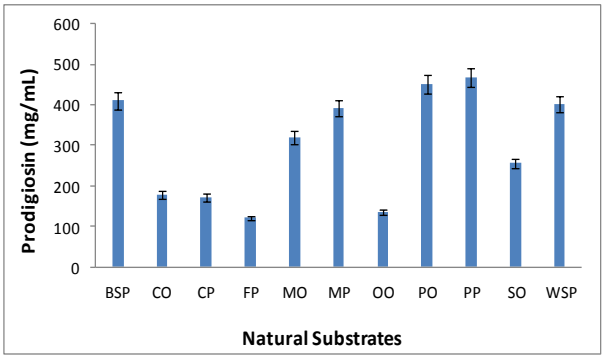

Fig.11:Screening of various natural substrates on production of prodigiosin by *S. marcescens* MBB05 (Nutrient broth; inoculum 5%; pH 7; temperature 30°C; incubation period 36 hours; results are mean of independent experiment ± SD and are expressed as mg/mL.

Screening of various media on production of prodigiosin using peanut powder

The potential selected substrate peanut powder was added to various media like nutrient broth, peptone glycerol broth and finally with distilled water alone. In this highest prodigiosin production of 509.4 mg/mL (*S. marcescens* MBB05), were recorded in distilled water added with 1.5% of peanut powder. In this study, least production of prodigiosin was 412.5 mg/mL in the peptone glycerol broth and nutrient broth containing 1.5 % of peanut powder, inoculated with the selected bacterial strain.

Effect of various concentration of peanut powder on prodigiosin production

Peanut powder at various concentrations (0.5 to 4.0%) was tested. Highest prodigiosin production was 560.4 mg/mL recorded at the concentration of 2.0 % Peanut powder. Sudden rise in prodigiosin production was noted from 350.7 to 560.4 mg/mL at 1.5 to 2.0% substrate concentration levels respectively (Fig.12). Very low production of 155.0 mg/mL recorded at 0.5% of peanut powder. In terms of yield peanut medium has given the maximum of ~39 mg/mL[19]. The role of saturated fatty acid is that peanut has a higher concentration than sesame and the yield of prodigiosin is also higher in powdered peanut broth than in powdered sesame broth. In the present study, it was apparent that the influence of these physico-chemical parameters to some extent could improve the production of prodigiosin. The peanut powder in distilled water, a media for producing prodigiosin is a promise for higher yield.

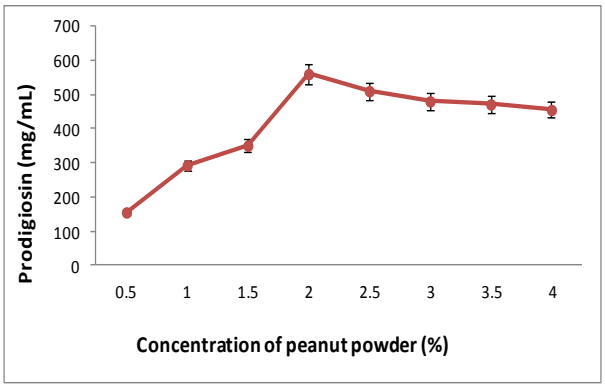

Fig.12:Effect of various concentration of peanut powder on production of prodigiosin by *S. marcescens* MBB05 (Distilled water; inoculum 5%; temperature 30°C; incubation period 36 h; results are mean of independent experiments ± SD and are expressed as mg/mL.

CONCLUSION

Biopigments produced by bacteria possess enormous efficiency as medicinally important products. We have been successful in designing a economically feasible medium supporting the enhanced growth of *Serratia marcescens* and simultaneously supporting a high yield of medicinally important biopigment prodigiosin. The selected bacterial strain isolated from Western Ghat Ecosystem was identified as *Serratia marcescens* and named as *Serratia marcescens* MBB05 based on the morphological and 16SrRNA gene sequence. The optimum condition for prodigiosin production was attained when incubated at 30°C, with a pH of 7.0, at 36 h of incubation with 5.0% inoculum supplemented with glucose, yeast extract and cysteine as best carbon, nitrogen and amino acid source for the selected bacterial strains. Prodigiosin production using basal media at optimized condition showed 1.7 times higher than the standard media and among the substrates tested, peanut powder was found to be the best natural substrate at a concentration of 2.0% in distilled water. The production was 4.5 times higher than the optimized basal medium. An economically cheaper media formulated for prodigiosin production would be a boon to pharmaceutical industries for large scale production of the medicinally potential drug, prodigiosin.

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