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COST EFFECTIVE PRODUCTION OF PRODIGIOSIN BY NATIVE ISOLATE SERRATIA MARCESCENS MBB01

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ABSTRACT

A new strain of *Serratia marcescens* MBB01, the natural red pigment prodigiosin producing strain was isolated from Western Ghat Ecosystem, Tamil Nadu, India. Prodigiosin, a natural pigment synthesized by bacteria is gaining much interest because of their huge competence as medicinally important product. The regular liquid media currently being used for prodigiosin biosynthesis are nutrient broth, peptone glycerol broth and production medium. A natural media which support 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. For this, eleven natural substrates have been tested. Among the tested substrates, peanut powder was found to be the best natural substrate at a concentration of 2 % in distilled water; pH 7; inoculum (5%); temperature (30°C); incubation period of 36 h. The production was 5.2 times higher (470.6 mg/mL) than the optimized basal medium.

KEYWORDS: **prodigiosin; *Serratia marcescens*; natural substrate; peanut powder**

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INTRODUCTION

Secondary metabolites have proven to be an unlimited source of biologically active molecules and some of these bioactive compounds are used in clinical or veterinary medicine or as tools for biological research[1]. Prodiginines are classical secondary metabolites only appearing in the later stages of bacterial growth[2]. It is produced by *Serratia marcescens*, *Pseudomonas magnesorubra*, *Vibrio psychroerythrous* and other bacteria. The red pigment of *Serratia marcescens*, prodigiosin, has interested chemists because it has been reported to be the only naturally occurring tripyrrylmethene[3, 4]. Owing to the characteristic red color of these secondary metabolites, colonies of the pigment producing *S. marcescens* strikingly resembled droplets of blood. This was linked with many seemingly miraculous (prodigious) events and the pigment was, therefore, named as prodigiosin. The term "prodigiosin" is used in the literature in a generic sense to include a family of similar compounds. Prodigiosin has no defined role in the physiology of producing strains, but it has been suggested to have a role in metabolic overflow. Prodigiosins are a family of naturally occurring tripyrrole ring-containing red pigments having a common pyrrolyldipyrrolylmethene skeleton. These pigments are emerging as a novel group of compounds having distinct biological activities (antibacterial, antimalarial, antimycotic, immunomodulating, antitumor and nuclease[3, 5, 6]. It may also be an important factor for the trypanolytic activity of*S. marcescens*[7]. 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[8, 9]. It forms lustrous, square pyramidal crystals that are dark red with a green reflex. The highly conjugated system of seven double bonds presumably accounts for the intense pigmentation[10]. Prodigiosin is synthesized as a secondary metabolite by biotypes A_1, A_2 and A_6 of *Serratia marcescens* and by some strains of other taxonomically unrelated bacteria[9]. Sesame seed to give a better yield in terms of prodigiosin biosynthesis further comparison was done with readily available cheaper sources like peanut and coconut. Sesame oil, peanut oil and coconut oil were also compared with the rest of the media[11, 12]. The maximum prodigiosin production was seen at 28 and 30°C in nutrient broth. 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[13]. Designing a new, nutritious and economically cheap medium was thought for the production of prodigiosin. Bacteria possess huge ability in producing biopigments that are synthesized for producing medicinally important products. 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. 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[14]. In 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 to test their efficiency in fulfilling our aim.

MATERIALS AND METHODS

Isolation and screening of bacteria for prodigiosin production

Soil samples were 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 2-7 days and stored in a sealed plastic container before use. One gram of soil sample was serially diluted (10^{-2} to 10^{-7}) with sterile distilled water

and spread plated on nutrient agar (g/L) (peptone: 5, beef extract: 3, yeast extract: 2, sodium chloride: 5; agar: 1.5; pH: 7.0 ± 0.2) plates. Four 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, 100 mL conical flasks containing 50 mL of nutrient broth (g/L) was prepared and used.

Forty eight 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 30 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 2 mL of chloroform and transferred into fresh sterile micro tubes. It was again evaporated at room temperature to concentrate the pigment[15].

Estimation of prodigiosin

The acidified prodigiosin was measured at 535 nm using spectrophotometer. Prodigiosin yield was measured from a calibration curve based on the A535 of standard prodigiosin[15].

Isolation of chromosomal DNA

The template DNA (selected bacterial strains) for the PCR amplification was isolated by modification of the methods suggested by Cook and Meyers[16] and that of Coombs and Franco[17].

Molecular identification

The isolate was further confirmed at the species level based on the 16s rRNA sequence alignment. PCR amplification 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*, as described by Weisburg *et al.*[18].

Sequence comparison and phylogenetic analysis

The 16S rRNA gene sequence of the isolate was compared with available 16S rRNA gene sequences in GenBank databases using the BLAST search facility at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/).

Optimization studies

Effect of various media on prodigiosin production

The 100 mL conical flasks containing 50 mL of nutrient broth (g/L) (peptone: 5; beef extract: 3; yeast extract: 2; sodium chloride: 5; pH: 7.0 ± 0.2) and peptone glycerol broth (g/L) (meat extract 10; peptone 10; glycerol 10; pH: 7.0 ± 0.2) was prepared and sterilized separately. Forty eight hours old inoculum (5%) was added to each of the flasks and incubated at 27°C in a sterile condition. The incubation period broth was taken for pigment extraction and estimation.

Effect of various physico-chemical parameters on prodigiosin production

Having an insight on the composition of already published media the idea of designing a new, nutritious and economically cheap medium was thought of for the prodigiosin biosynthesis. In order to 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. Keeping these objectives in mind, a media which supports the growth of the bacteria and at the same time prove efficient to activate high levels of pigment formation was planned.

Influence of temperature

Investigations on the effect of cultivation temperatures on prodigiosin production have been carried out by incubating the nutrient broth at different temperatures. 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. The optimum temperature achieved by this step was fixed for subsequent experiments.

Effect of incubation time

To determine 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.

Optimization 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.

Influence of pH

In order to study the effect of pH of the nutrient broth on prodigiosin production, experiments were performed with different pH media. While optimizing the pH of the basal medium, the pH of aqueous solution 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

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

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.

Effect of different amino acids

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

Various concentrations of glucose, dried yeast and cysteine (0.25, 0.5 and 0.75 %) were added to the sterile nutrient broth separately. Forty eight hours 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

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 productionof prodigiosin using peanut powder

The 100 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) were prepared and sterilized separately. Fourty eight hours 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. The best substrate obtained at this level used for further experimental design.

Effect of various concentration of peanut powder on prodigiosin production

The 100 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. Forty eight hours 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 bacteria for prodigiosin production

Out of 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 MBB01 with 90.5 mg/mL of prodigiosin and 157 mg/mL of protein. Based on their highest prodigiosin production the bacterial strain MBB01 (90.5 mg/mL) was selected for further study. Gargallo-viola *et al*.[19] have said that among 105 *Serratia* spp. isolated from clinical and environmental sources, 28 produced prodigiosin. In his report stated that the seventy-four of the eighty-seven clinical isolates were unable to produce prodigiosin. The four bacterial strains were isolated from forest soil area, where one bacterial strain produced prodigiosin. Cang *et al.*[15] has also isolated an ethanol utilizing *Serratia marcescens* S389 from the soil sample, produced prodigiosin up to 3 mg/mL.

Molecular identification

PCR amplification and sequencing of the 16SrRNA genes

The isolate was further confirmed for the species level by using primers specifically designed for the identification of *Serratia marcescens* based on the 16s rRNA sequence alignment of soil isolates.

Nucleotide sequence accession number

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 GU290196, for *S. marcescens* MBB01. Ajithkumar *et al.*[20] have reported that the level of DNA–DNA hybridization between strain *S. marcescens* KREDT and *S. marcescens* JCM 1239T were found to have 97% similarity. Deorukhkar *et al.*[21] have isolated a strain that was found to be 88% similar to *S. marcescens* (AJ233431). The profile of the selected stain was also similar to *S. marcescens.*

Production of prodigiosin

Effect of different media on prodigiosin production

Two media were tested for prodigiosin production, *viz.,* nutrient broth and peptone glycerol broth. *S. marcescens* MBB01 showed prodigiosin production 112.1 mg/mL compared to 85 mg/mL in peptone glycerol broth. Extra cellular protein production of nutrient broth showed 46.4 mg/mL and 88.9 mg/mL 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.*[13] have 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 present study the selected strain has produced 112.1 – 166.3 mg/mL, which was much higher than the earlier report. 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. Peptones also contain small amounts of various organic and inorganic compounds. But they may be deficient in certain minerals and vitamins. 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 powder. These extracts are frequently used as a source of amino acids, vitamins and coenzymes and growth factors by fastidious organisms. Trace elementslike minerals and usually some sugar are also present. In peptone glycerol broth, the glycerol was the carbon source. 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[13].

Effect of various physico-chemical parameters for prodigiosin production

Production of pigment 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 studies 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

Temperature plays a very crucial role in the pigment production. In this optimization study, 27, 28, 30, 32 and 37°C were the temperatures selected. There was less prodigiosin production when the nutrient broth were 110.2, 113.1, 115.9, 21.2 mg/mL (*S. marcescens* MBB01), 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 129.5 mg/mL by *S. marcescens* MBB01 (Fig.1). Anuradha *et al.*[13] reported that 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.

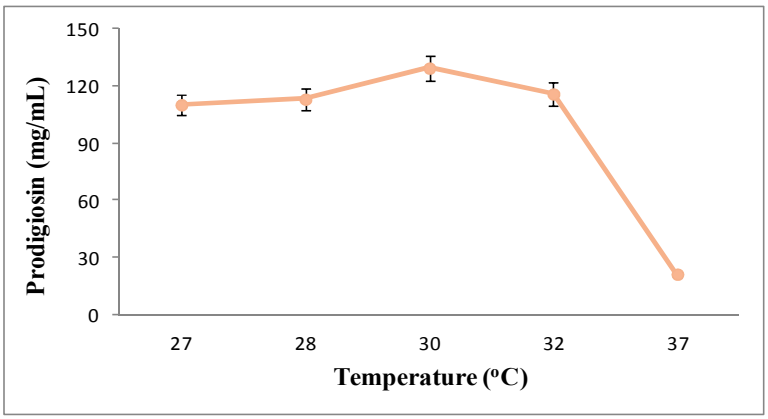

Figure.1.

Effect of temperature on production of prodigiosin by S. marcescens MBB01 (Nutrient broth; inoculum 5%; temperature 30°C; incubation period 36 h; results are mean of independent experiments ± SD and are expressed as mg/mL.

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 colour. Pryce and Terry,[22] reported that in nutrient broth and peptone glycerol broth, the prodigiosin production was completely blocked at 37°C. These reports are in agreement with the present finding. This might be due to the fact that the terminal step in prodigiosin biosynthesis *ie*. Condensing of mono and bipyrrole moieties was temperature sensitive[23]. Williams *et al.*[24] reported that the maximal amounts of prodigiosin were synthesized in either minimal or completed medium after incubation of cultures at 27°C for 7 days.

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, whereas at 12, 24, 36 and 48 h it ranges between 110 to 177 mg/mL.

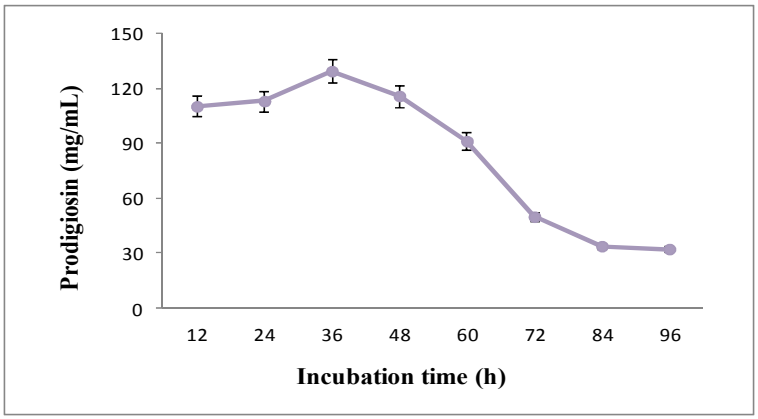

Figure.2.

Effect of Incubation time on production of prodigiosin by S. marcescens MBB01 (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 130 mg/mL on the 36 h of incubation, which was the maximum compared to all other incubation timings (Fig.2). Williams *et al.*[24] reported that 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, whereas prodigiosin did not reach a maximum until 96 h. Cang *et al.*[15] reported that the maximum prodigiosin production was noticed at 48 h and the production was complete by 72 h.

Effect of inoculum size

In order to further increase the prodigiosin production, the effect of various inoculum sizes was 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. The maximum prodigiosin production 115.1 mg/mL was observed at 5.0 % for *S. marcescens* MBB01 (Fig.3). Prodigiosin production was comparatively less at 2.5 and 7.5 % inoculum concentration.

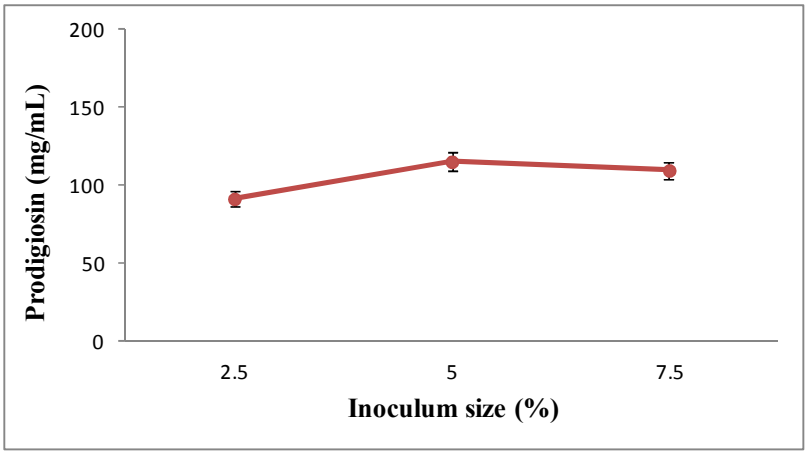

Figure.3.

Effect of Inoculum size (%) on production of prodigiosin by S. marcescens MBB01 (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 bacterial strains 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 was 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). Based on this study, *S. marcescens* MBB01 has produced 143.6 mg/mL which was the highest production recorded in the pH 7.0. Hence pH 7 was maintained in optimization studies.

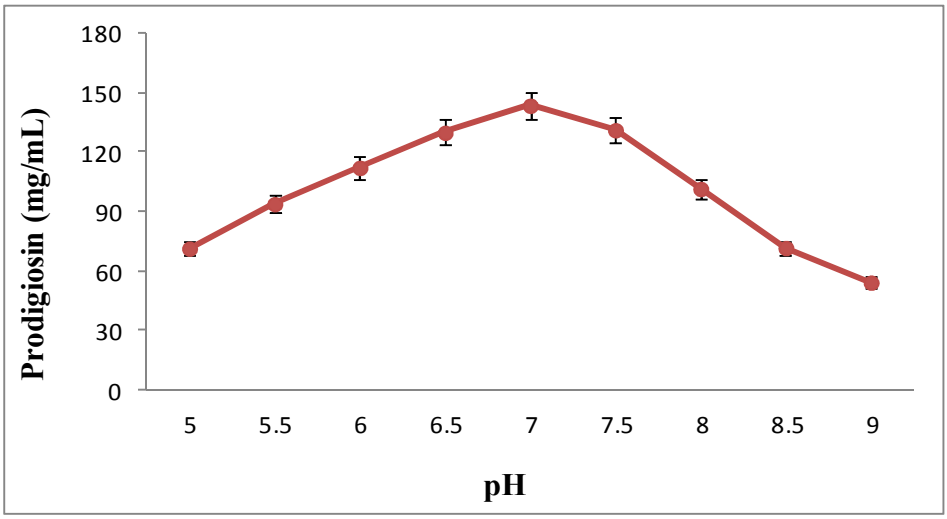

Figure.4.

Effect of pH on production of prodigiosin by S. marcescens MBB01 (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 influence of external pH on the regulation of biosynthesis of secondary metabolites has been described previously[25, 26, 27, 28]. In some cases, the optimum for biomass yield was different from that of metabolite production[26, 27]. *Serratia marcescens* was considered to grow optimally at pH 6.4-7.4. Sole *et al.*[29] reported that 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, pigment production is pH-dependent and the optimum pH is in the range 8.0-8.5. Woods *et al.*[30] reported that 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. However, cultures of*S. marcescens* have a powerful buffering capacity. Ruis *et al.*[31] and Williams and Qadri[9] have reported that irrespective of initial pH of media, the final pH was 7.2 to 8.0 as the bacteria grow. In our present study, the maximum prodigiosin was noticed in the medium at the pH of 7.0.

Role of different carbon sources

To evaluate 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 strain. Effect of ethanol, glucose and glycerol as a carbon source has greatly influenced the selected strain for the prodigiosin production. Prodigiosin production was 151.7 mg/mL for ethanol, 161.4 mg/mL for glucose and 153.7 mg/mL for glycerol inoculated with *S. marcescens* MBB01 (Fig.5). Less production was noticed in the case of lactose supplemented medium. Since *Serratia* sp. are non lactose fermenter the growth and other parameters would have reduce the pigment production.

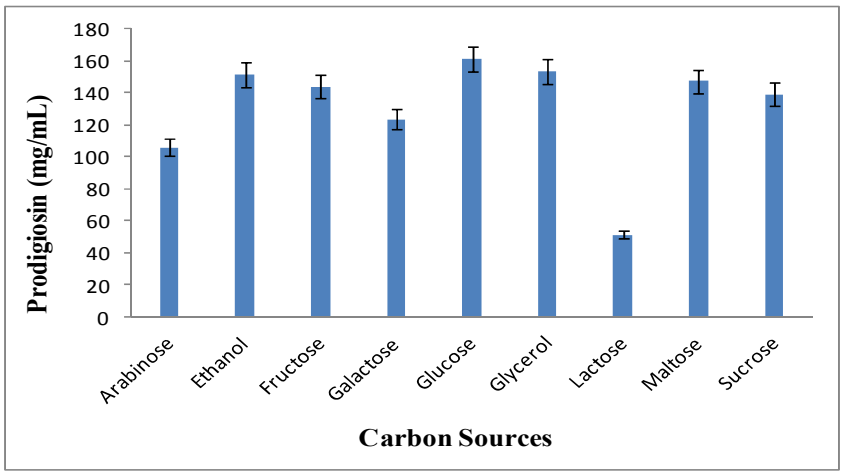

Figure.5.

Effect of Carbon sources on production of prodigiosin by S. marcescens MBB01 (Nutrient broth; inoculum 5%; temperature 30°C; incubation period 36 h; results are mean of independent experiments ± SD and are expressed as mg/mL.

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[32] and the media used had dextrose in the culture broth and casein in production medium. Cang *et al.*[15] have quoted a medium containing ethanol and carbon source but the yield was 3 mg/mL. 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.*[33] have 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.*[15] have 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[34]. Cang *et al.*[15] reported that the isolate *Serratia marcescens* S389 produced about 3 mg/mL of prodigiosin when grown on ethanol under the appropriate conditions. In the present study the maximum prodigiosin was noticed in the medium containing glucose as the substrate.

Effect of various concentrations of glucose

Glucose concentration of 0.5% was optimum for the production of prodigiosin. *S. marcescens* MBB01 has produced 196.2 mg/mL of prodigiosin at 0.5% concentration of glucose. At 0.25 and 0.75% concentration of glucose the production was 174.2, 103.6 mg/mL respectively (Fig.6). 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 substrates[35], this has been referred to as 'catabolite repression'.

In many secondary metabolite pathways, the enzymes subject to control by the carbon source are known[36, 37]. Prodigiosin production is inhibited when glucose is added to the growth medium[9, 38]. This substrate inhibits the synthesis of the pigment in cultures grown on solid medium with concentrations up to 15g/L, and there is close correlation between glucose consumption and the synthesis of this secondary metabolite[38]. 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[39, 40]. 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[41]. However, cAMP does not appear to be involved in the glucose effect, on the synthesis of some secondary metabolites[25, 42, 43].

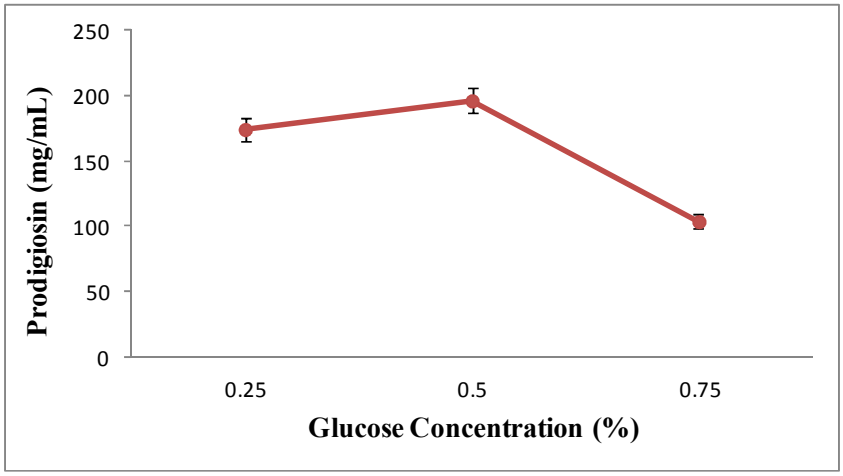

Figure.6.

Effect of various glucose concentration on production of prodigiosin by S. marcescens MBB01 (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

Nitrogen source is essential for the organism for prodigiosin production. Different nitrogen sources *viz*., ammonium chloride, ammonium nitrate, ammonium sulphate and dried yeast were tested. Dried yeast supported the three selected isolates for maximum prodigiosin pigment production. Strain *S. marcescens* MBB01 produced 161.4 mg/mL of prodigiosin. 83.3, 71.1 and 54.5 mg/mL prodigiosin was produced when ammonium chloride, ammonium nitrate and ammonium sulphate was used respectively (Fig.7). Cang *et al.*[15] has studied various nitrogen sources for prodigiosin production, in that pharmamedia and polypepton gave a good antibiotic production as well as good bacterial growth and they also state 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, and it seems that the promotion of prodigiosin production by strain *Serratia marcescens* S389 under phosphate limitation is reasonable[44]. 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.

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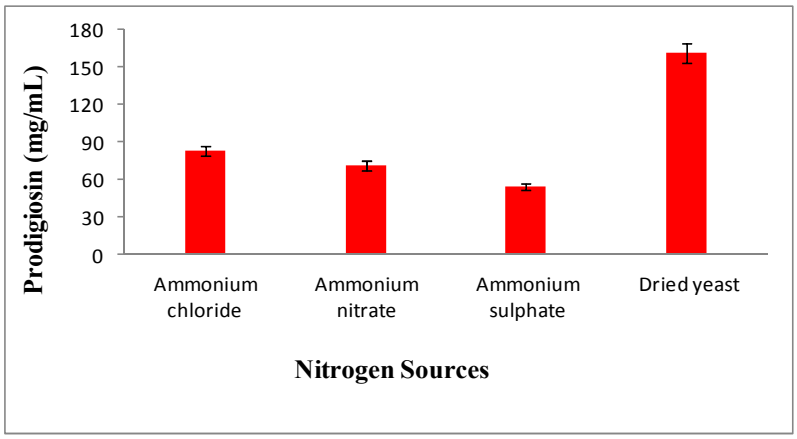

Figure.7.

Effect of Nitrogen sources on production of prodigiosin by S. marcescens MBB01 (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

Different amino acid sources, proline, methionine, tryptophan, leucine and cysteine were tested. Cysteine containing nutrient broth media supported maximum prodigiosin pigment production (168.1 mg/mL). The next best amino acid was proline that produced 155.1 mg/mL (Fig.8). 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. Scott *et al.*[45] demonstrated that proline did not cause biosynthesis of prodigiosin in non-proliferating cells unless it was catabolized. Proline oxidase is the first enzyme in proline degradation and its activity rises during the first 8 hrs 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.

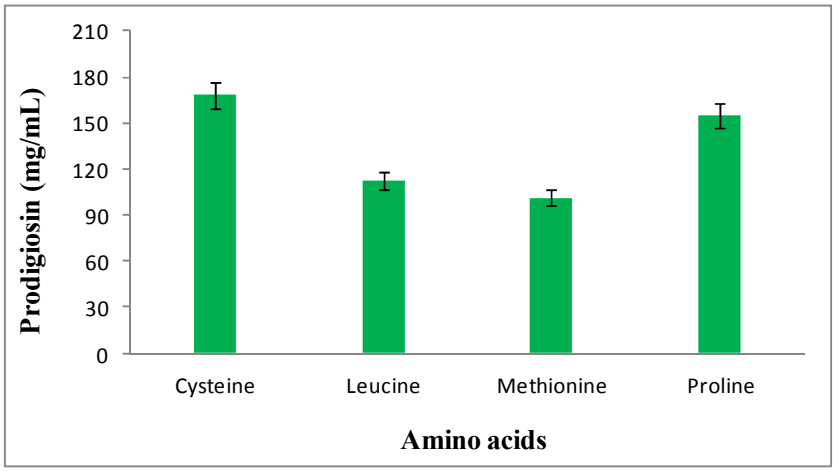

Figure.8.
Effect of Amino acids on production of prodigiosin by S. marcescens MBB01 (Nutrient broth; inoculum 5%; temperature 30°C; incubation period 36 h; results are mean of independent experiments ± SD and are expressed as mg/mL.

Only amino acids were utilized as sources of both carbon and nitrogen for growth, resulted in biosynthesis of prodigiosin in nonproliferating cells of *S. marcescens*[46]. After the 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 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[47]. 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[46]. Tanaka *et al.*[48] and Wasserman *et al.*[49] have reported that the ring of proline entered intact into the bipyrrole part of the prodigiosin molecule.

Effect of various concentrations of dried yeast extract

Yeast extract concentration of 0.5% was optimum for the production of prodigiosin. *S. marcescens* MBB01 has produced 166.2 mg/mL of prodigiosin at 0.5% concentration of yeast extract (Fig.9).

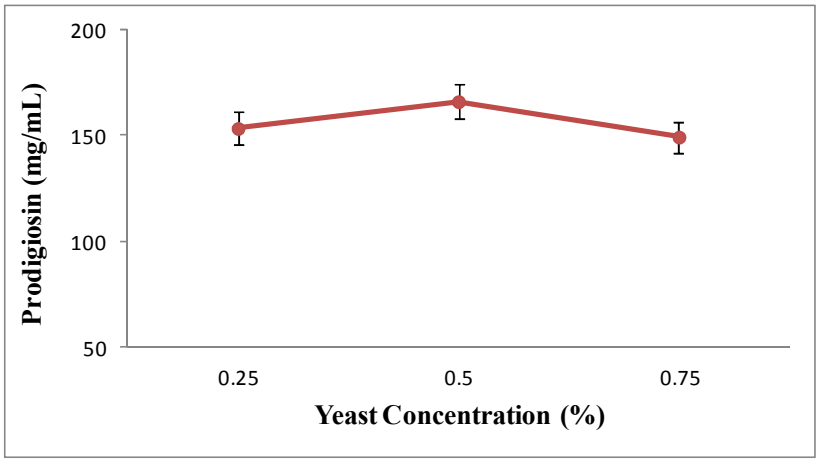

Figure.9.

Effect of various yeast concentration on production of prodigiosin by S. marcescens MBB01 (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 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* MBB01, the highest prodigiosin production was seen in peanut powder as substrate, with the production of 371.1 mg/mL respectively (Fig.5). It is also noted that olive oil ranked low in prodigiosin production, 121.5 (*S. marcescens* MBB01). MBB01 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. Anuradha *et al.*[13] have studied that the oils are known for their high levels of unsaturated fatty acid content and a very low percentage of saturated fatty acids. From the results observed by them the pigment yield was 15 times more in media containing fatty acid seeds than in oils. According to Kim *et al.*[50], oil gave a better yield over the various carbon (not fatty acid containing seeds) and nitrogen sources tested. Anuradha *et al.*[13] have also said that 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.

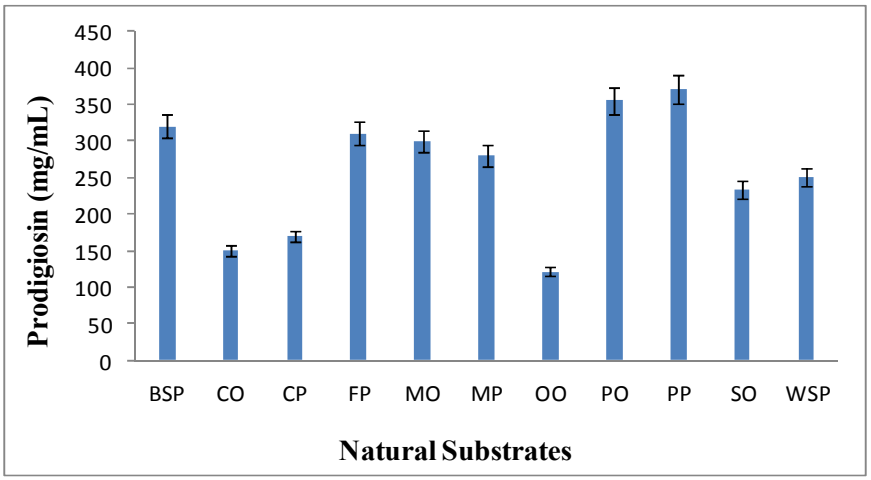

Figure.10.
Screening of various natural substrates on production of prodigiosin by S. marcescens MBB01 (Nutrient broth; inoculum 5%; pH 7; temperature 30°C; incubation period 36 hours; results are mean of independent experiment ± SD and are expressed as mg/mL.

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*. Nakamura[51] in his patent describes the use of sodium oleate media and the substitution of sodium oleate with oleic acid. The bonded fatty acids as carbon source are less accessible by *Serratia marcescens*[52]. 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. Hence peanut powder has been selected for further studies using different media.

Screening of various media on production of prodigiosin using peanut powder

The 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 442.4 mg/mL (*S. marcescens* MBB01) was recorded in distilled water added with 1.5% of peanut powder. In this study least production of prodigiosin was 384.0 and 395.2 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 concentrations of cysteine

Cysteine concentration of 0.5% was optimum for the production of prodigiosin. *S. marcescens* MBB01 has produced 171.5 mg/mL of prodigiosin at 0.5% concentration of cysteine (Fig.11).

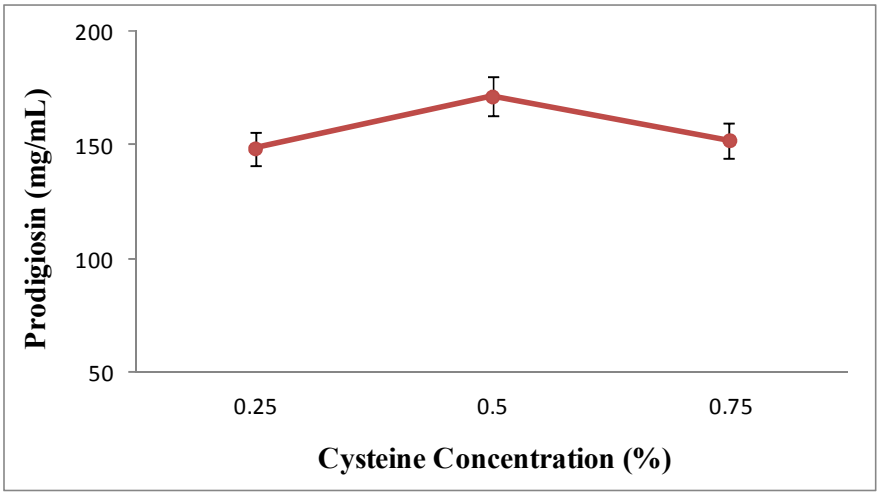

Figure.11.

Effect of various cysteine concentration on production of prodigiosin by S. marcescens MBB01 (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 concentration of peanut powder on prodigiosin production

Peanut powder at various concentrations (0.5 to 4.0%) was tested. Highest prodigiosin production was 470.6 mg/mL recorded at the concentration of 2.0% peanut power. Sudden rise in prodigiosin production was noted from 384.1 to 470.6 mg/mL at 1.5 to 2.0% substrate concentration levels respectively (Fig.12). Very low production of 34.1 mg/mL was recorded at 0.5% of peanut powder.

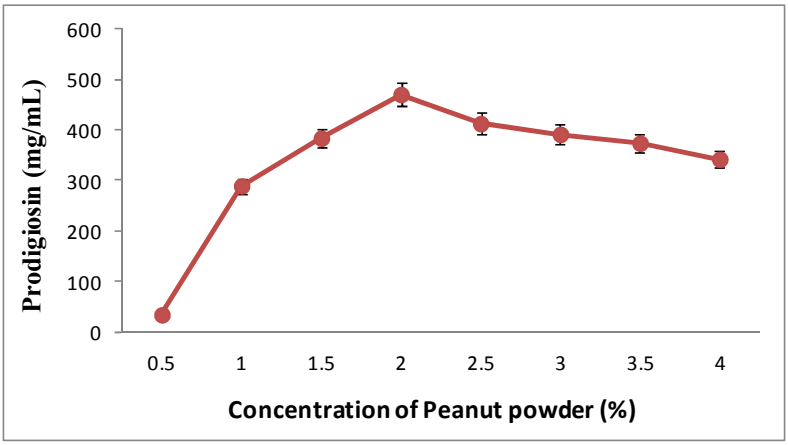

Figure.12.

Effect of various concentration of peanut powder on production of prodigiosin by S. marcescens MBB01 (Distilled water; inoculum 5%; temperature 30°C; incubation period 36 h; results are mean of independent experiments ± SD and are expressed as mg/mL.

Anuradha *et al.*[13] reported that the yield peanut medium has given the maximum of ~39 mg/ml. 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. Premalatha *et al.*[53] reported that the maximum naphthoquinone

pigment production was (124.8 µM/g) by *Fusarium moniliforme* MTCC6985 using different medium. 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.

CONCLUSION

Prodigiosin, a natural pigment synthesized by bacteria are gaining much interest because of their huge competence as medicinally important product. The selected bacterial strain isolated from Western Ghat Ecosystem was identified as *Serratia marcescens* 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 1.9 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 5.2 times higher than the optimized basal medium. A novel strain producing prodigiosin has been discovered which could be exploited for industrial purpose. 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. In addition, a number of lead compounds have emerged in preclinical and phase I/II clinical trials, demonstrating prodigiosin as not only of academic interest but also an attractive anticancer agents.

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