INTERNATIONAL JOURNAL OF ADVANCED BIOLOGICAL RESEARCH

www.scienceandnature.org

STUDY ON OPTIMIZATION OF PRODIGIOSIN PRODUCTION BY *SERRATIA MARCESCENS* MSK1 ISOLATED FROM AIR

Mohammed Husain Bharmal, Naseer Jahagirdar & Aruna K.
Department of Microbiology, Wilson College, Mumbai-400007, India

ABSTRACT
Prodigiosins, a family of natural red pigments characterized by a common pyrrolylpyrromethane skeleton, producing bacterium was isolated from air and identified as *Serratia marcescens*MSK1 based on the morphological, cultural, biochemical tests and 16S rRNA gene sequence analysis. Prodigiosin production was induced only by methionine or cysteine in the presence of glucose by this isolate. Prodigiosin production was optimized to obtain maximum yield in M9 medium with 0.4% mannose, 0.01% Methionine, 0.003% Cysteine and 0.1% Ammonium chloride, pH 8 at 28^0C under shaker conditions (120rpm) for 24 hrs. Pigment also exhibited antibacterial activity against gram positive microorganisms.

KEYWORDS: *Serratia marcescens* MSK1, Prodigiosin, pyrrolylpyrromethane.

INTRODUCTION

Prodigiosin has been known to be a natural compound showing a broad range of cytotoxic, antifungal, antibacterial, algicidal, antiprotozoal, antimalarial, immunosuppressive, anticancer and antiproliferative activities (Furstner, 2003; Samrot *et al*., 2011; Cerdeno *et al*., 2001) and is produced by *Vibrio psychroerythrus* (D'Aoust and Gerber, 1974), *Serratia marcescens*, *Pseudomonas magnesiorubra,* and other eubacteria (Lewis and Corpe, 1964). *S. marcescens* is the major producer of Prodigiosin (Furstner, 2003). Prodigiosin {5[(3-methoxy-5-pyrrol-2-ylidene-pyrrol-2-ylidene) –methyl] -2- methyl-3-pentyl-1H- pyrrole} is an alkaloid secondary metabolite with a unique tripyrrole chemical structure, first characterized from *S. marcescens,* a Gram negative bacterium which is an opportunistic pathogen with the nonchromogenic biotypes posing a public health threat (Kalesperis *et al.*, 1975; Gargallo *et al.*, 1987; Gargallo,1989). Chromogenic biotypes from the natural environment have rarely implicated in infections and the function of this red pigment remains unclear because clinical isolates are rarely pigmented (Hejazi and Falkiner, 1997). Interestingly, the water insoluble red pigment produced by *S. marcescens* has been reported to have antibiotic activity(Tsuji et *al.*, 1990; Kataoka et *al.*, 1992; Tsuji et *al.*, 1992; Songia *et al.,*1997).Some strains of *S. marcescens* also produce a water-soluble, reddish-violet pigment with superoxidase dismutase mimetic activity (Hardjito *et al.*, 2002).

The production of Prodigiosin in *S. marcescens* is susceptible to temperature and is substantially inhibited at temperatures higher than 37^0C (Giri *et al*., 2004). Conventional media used for the biosynthesis of Prodigiosin by *S. marcescens* strains are complex media that are rich in a variety of nutrients (Furstner, 2003; Giri *et al*., 2004; Yamashita *et al*., 2001**).** Certain nutrients such as thiamine and ferric acid are particularly crucial for Prodigiosin production, whereas phosphate adenosine triphosphate and ribose have inhibitory effects on Prodigiosin yield (Wei and Chen, 2005; Witney *et al*., 1977; Lawanson and Sholeye, 1975). Giri *et al.* (2004) tested the performance of a series of media and discovered that a novel peanut seed broth give rise to a significant enhancement of Prodigiosin production.

The biosynthesis of the pigment is a bifurcated process in which mono and bipyrrole precursors are synthesized separately and then assembled to form Prodigiosin (Boger and Patel, 1988). The biosynthesis of Prodigiosin by *Serratia marcescens* has been examined by the incorporation of labeled precursors (Wasserman *et al*., 1973). A complete pathway for prodiginine biosynthesis has been deduced by analysis of the red cluster in *Streptomyces coelicolor*A3 (2), and the studies demonstrated that undecylprodiginine is derived from one unit of proline, one unit of glycine, one unit of serine and several units of acetate, via a convergent pathway involving condensation of 4-methoxy-2,2P bipyrrole-5-carboxaldehyde and 2-undecylpyrrole at a late stage. The macrocyclic prodiginines appear to be derived from undecylprodiginine by oxidative cyclisation (Cerdeno *et al*., 2001; Khanafari *et al*., 2006). Each pyrrole ring is constructed in a different way. Recent work using 13C-labeled precursors and Fourier transform 13C nuclear magnetic resonance has shown the pattern of incorporation for acetate, proline, glycine, serine, alanine and methionine into Prodigiosin (Gerber**,** 1975).

Kobayashi and Ichikawa (1991) and Matsuyama *et al.* (1986) reported that Prodigiosin is associated in extracellular vesicles or present in intracellular granules. Most pigments absorb light at some defined wavelength, and pigment expression may be easily monitored spectrophotometrically (Cerdeno *et al*., 2001). Prodigiosin can exist in two distinct forms, depending upon the hydrogen ion concentration of the solution. In an acid medium the pigment is red and exhibits a sharp spectral peak at 535 nm. In an alkaline medium the pigment is colored orange-yellow and possesses a broader spectral curve centered at 470 nm (Williams *et al.,* 1955).

The current study focuses on isolation and identification of Prodigiosin producing organism from air, study of effect of different amino acids on Prodigiosin production and optimization of Prodigiosin yield, chromatrographic and antibacterial studies of Prodigiosin are further discussed in this paper.

MATERIALS AND METHODS

Isolation, screening and identification

Bacteria were isolated on Nutrient agar plates (Peptone: 10g/l, Meat extract: 3g/l, Sodium Chloride: 5g/l, pH-7, Agar: 22g/l) from various sources viz. soils, swabs from leaves and sampling of air from laboratory which was carried out by keeping nutrient agar plates exposed to air for 30 minutes. The plates were incubated at 28^0C for 24 hrs. The morphologically distinct bacterial strains showing orange to maroon color colonies were selected for further study and were maintained on nutrient agar slant at 4^0C.

Presumptive colour tests for Prodigiosin was carried out by scraping the pigmented growth on Nutrient agar medium plates and suspending overnight in 95% ethanol at 22 to 24°C. Debris was removed from the suspension by centrifugation at 5,000 x g for 15 min. The clear solution was then divided into two portions. One part was acidified with a drop of concentrated HCl; the other part was alkalinized with a drop of concentrated ammonia solution. A red or pink color in the acidified solution and a yellow or tan color in the alkaline solution indicated a positive, presumptive test for Prodigiosin (Ding and Williams, 1982). Identification of the isolate was done on the basis of morphological, cultural and biochemical tests' using Bergeys Manual of Determinative Bacteriology 8th edition (Buchanan and Gibbons, 1975) and the strain was confirmed by 16S rRNA gene sequence analysis carried out by Xcelris Lab, Ahmadabad.

Pigment extraction

The pigment was extracted by adding 4 volumes of acetone to the cell suspension. The acetone mixture was shaken for 3 hrs at room temperature, and then centrifuged. The sedimented cell debris was washed twice by resuspending in 50 ml. of acetone, shaking for 30 min followed by centrifugation. The washings were combined with the supernatant from the original centrifugation, and the solution was filtered. Pigment was extracted from small portions of the filtrate by mixing thoroughly 1 volume of the acetone solution with 2 volumes of petroleum ether in a separatory funnel. The acetone was removed by adding 10 volumes of water to the funnel, then drawing off the acetone-water phase. This procedure was repeated until the entire filtrate was extracted, and the pigment was in the petroleum ether phase. To obtain dry pigment, the petroleum ether extract was evaporated at 30^0C. The dry pigment was used for chromatographic or spectral analysis (Williams *et al*., 1955).

Determination of absorption spectra

Spectral analysis was made on dried pigment extracted by the above method by dissolving in 10 ml of absolute ethanol. Acidic conditions for spectral analysis were obtained by adding 1 ml of 1 N Hydrochloric acid, to 10 ml of the ethanol extract (Williams *et al*., 1955). Spectral analysis was made on a UV-visible spectrometer Systronics 2203 model.

Effect of amino acids on Prodigiosin production

To check effect of different amino acids, (Threonine, Lysine, Leucine, Tryptophan, Tyrosine, Glutamate, Glutamine, Aspartate, Aspargine, Proline, Phenylalanine, Cysteine, Isoleucine, Arginine, Methionine, Alanine, Histidine, Serine, Valine and Glycine) sterilized filter paper discs were dipped in different sterilized amino acid solutions (1%) and placed on M9 minimal salt agar medium (Disodium Hydrogen phosphate: 3.2 g/l, Potassium Dihydrogen phosphate: 3.0g/l, Sodium chloride: 5g/l, Ammonium chloride: 1g/l, pH: 7.4, Agar: 22g/l) with 0.5% glucose and swabbed with isolate (suspended in Phosphate buffer saline, pH-7.2) adjusted at 0.5 O.D. at 660nm.The relatively long wavelength was chosen for optical density measurements because it was not affected by the presence of Prodigiosin pigment (Pryce *et al.*, 2008). Plates were incubated at 28^0C for 24 hrs. Positive results were indicated by the presence of pigmented growth around the paper disc.

Optimization of Prodigiosin production

Assay of Prodigiosin production

Prodigiosin displays a characteristic absorption spectrum in acidified ethanol, with a strong maximum at 534 nm. Thus the relative concentration of Prodigiosin produced by liquid grown cultures was quantified as follows: 1 ml samples were harvested by centrifugation at 13000 rpm for 5 min. The supernatant was discarded and the pellet resuspended in acidified ethanol (4% 1 M HCl in ethanol) to extract Prodigiosin from the cells. Cell debris was removed by a second centrifugation step and the supernatant transferred to a cuvette for measurement of absorbance at 534 nm (Slater *et al*., 2003).

Optimization of amino acids

Effect of different concentrations of Cysteine (0.01-0.1% and 0.001-0.01%) and Methionine (0.01-0.1%) was studied, in M9 minimal salt broth medium containing 0.5% glucose, in order to find the optimum concentration of Cysteine and Methionine for Prodigiosin production. Flasks were incubated at 28^0C under shaker conditions for 24 hrs (Giri *et al*., 2004; Heinemann *et al*., 1970). Two different combinations of Methionine and Cysteine (0.01% Methionine and 0.003% Cysteine and 0.02% Methionine and 0.006% Cysteine) in M9 minimal medium with 0.5% glucose were also studied for maximum pigment production.

Effect of different carbon sources on Prodigiosin production

To study the effect of different carbon sources on Prodigiosin production 0.5% of different sugars (Arabinose, Xylose, Rhamnose, Glucose, Fructose, Mannose, Mannitol, Glycerol, Sucrose, Lactose, Galactose, Maltose and Raffinose), polysaccharide (Starch, Glycogen) and oils (Palmitic acid, Stearic acid, Til oil, Olive oil, Coconut oil, Palm oil, Mustard oil and Sunflower oil) were added in the M9 medium containing optimized concentrations of amino acids. All the flasks were incubated at 28^0C under shaker conditions for 72 hrs and Prodigiosin production was checked at interval of 24 hrs. Various concentrations (0.1-1%) of optimized carbon source were added in order to find out optimum concentration for Prodigiosin production.

Effect of different nitrogen sources on Prodigiosin production

The effect of different nitrogen sources on Prodigiosin production was studied by replacing 0.1% ammonium chloride in the M9 medium with 0.1% of different organic and inorganic nitrogen sources such as Ammonium chloride, Ammonium oxalate, Ammonium citrate, Ammonium nitrate, Ammonium sulphate, Ammonium dihydrogen phosphate, Diammonium hydrogen phosphate, Ammonium ferrous sulphate, potassium ferricyanide, Sodium nitrite, Potassium nitrate, 2-Nitrophenol, 4-Nitrophenol, 4- Dimethyl amino benzaldehyde, Diphenyl amine and Urea. All the flasks were incubated at 28^0C under shaker conditions for 24 hrs.

Effect of pH on Prodigiosin production

To find out effect of pH on Prodigiosin production, different pH values (7, 7.5, 8.0, 8.5, and 9.0) were adjusted using 1N NaOH in M9 minimal salt broth medium containing optimized concentrations of amino acids, C source and N source and the flasks were incubated at 28^0C under shaker conditions for 24 hrs.

Comparison of Prodigiosin production in optimized M9 medium with Nutrient broth and Luria Bertani broth

To find out efficiency of Prodigiosin production in optimized M9 medium, it was compared with Nutrient broth and Luria Bertani broth at 28^0C under shaker conditions for 24 hrs.

Chromatographic separation

In order to characterize the pigment, thin layer chromatography (TLC) was performed. In this the pigment extract was loaded on a silica gel plate (Silica gel was prepared by adding 35 gms of silica powder in 100 ml Distilled water) and then placed in a solvent chamber containing 7:3 ratio Petroleum ether and Acetone.

Antibacterial effect

To examine the antibacterial effect of the pigment, Ditch plate technique was used, since the pigment was insoluble. In this, a ditch of 1.5 X 7cm. was made in Mueller-Hinton agar (pH-7) at the centre and the pigment extracted was dispersed in water and then added to molten agar such that the final concentration was approximately 50µg/ml (dry weight of extracted Prodigiosin) and then poured into the ditch. Laboratory cultures (*Escherichia coli, Klebsiella pneumoniae. Staphylococcus aureus, S. aureus 6538p, Bacillus subtilis, Proteus vulgaris and P. mirabilis*) were then streaked perpendicular to the ditch and the plates were incubated at 28^0C for 24 hrs. Antibacterial effect was determined by observing presence or absence of growth on the ditch.

RESULTS

Isolation, screening and identification

Screening of the pigment producing organisms from different samples resulted in six isolates showing orange to maroon coloration. The promising isolate which showed Prodigiosin production on the basis of presumptive test was selected for present study, and was maintained on nutrient agar slant at 4^0C. The cultural, morphological and biochemical tests were used to identify this isolate as *Serratia marcescens*. From 16S rRNA result, it was confirmed that the culture was *Serratia marcescens* strain MSK1.

Absorption spectrum

In order to determine the wavelength at which maximum absorption occurs, spectrum scan was done using UV-visible spectrometer Systronic 2203 model. The spectrum scan results showed that the Prodigiosin molecule in acidic conditions showed an absorption maximum at 534 nm (Fig. 1).

Effect *of amino* acids on Prodigiosin production

Serratia marcescens strain MSK1 showed zone of pigmentation around the discs impregnated with Cysteine and Methionine individually in the presence of 0.5% glucose at 28^0C after 24 hrs as shown in figure 2 and figure 3.

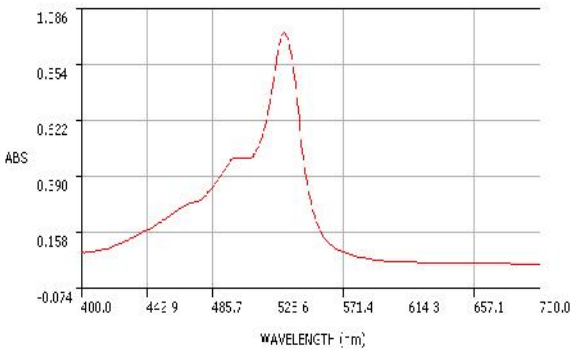

Figure 1: Spectrum scan of Prodigiosin produced by *Serratia marcescens* MSK1.

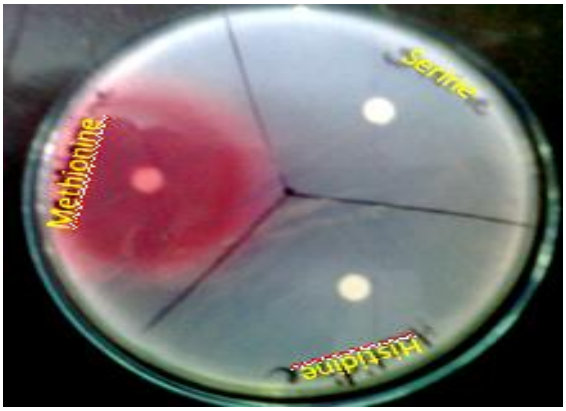

Figure 2: *Serratia marcescens* MSK1showing pigmentation in presence of Methionine

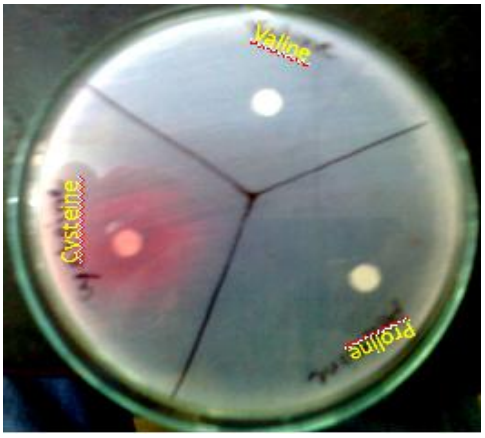

Figure 3: *Serratia marcescens* MSK1 showing pigmentation in presence of Cysteine

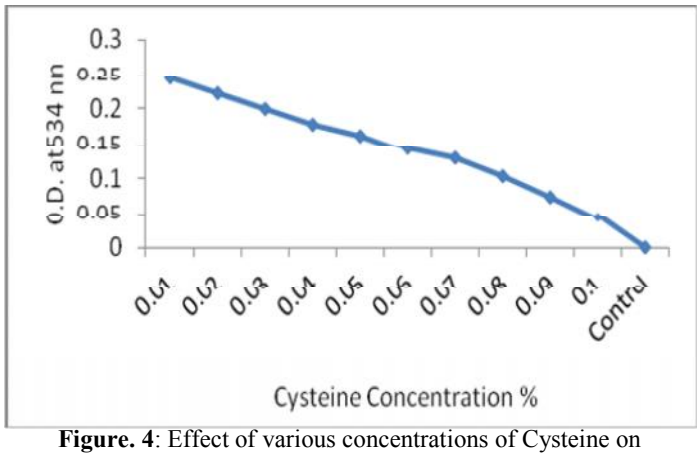

Figure. 4: Effect of various concentrations of Cysteine on Prodigiosin production by *S. marcescens* MSK1

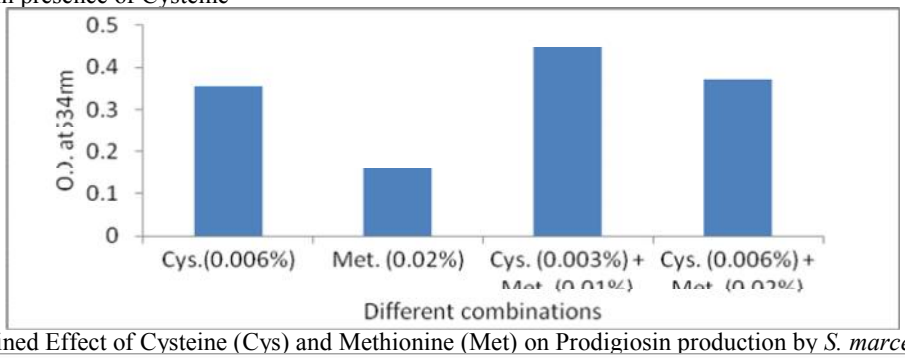

Figure 7: Combined Effect of Cysteine (Cys) and Methionine (Met) on Prodigiosin production by *S. marcescens* MSK1

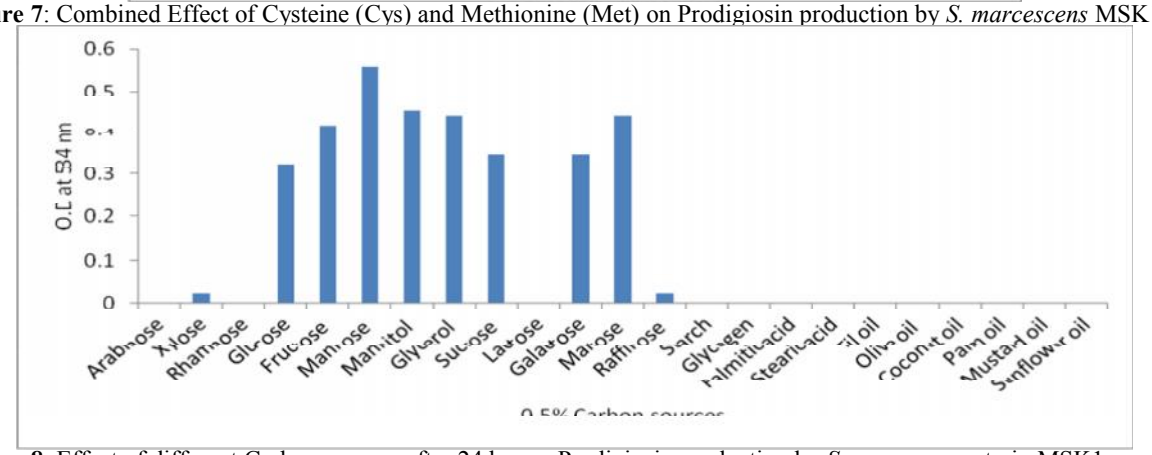

Figure 8: Effect of different Carbon sources after 24 hrs on Prodigiosin production by *S. marcescens* strain MSK1

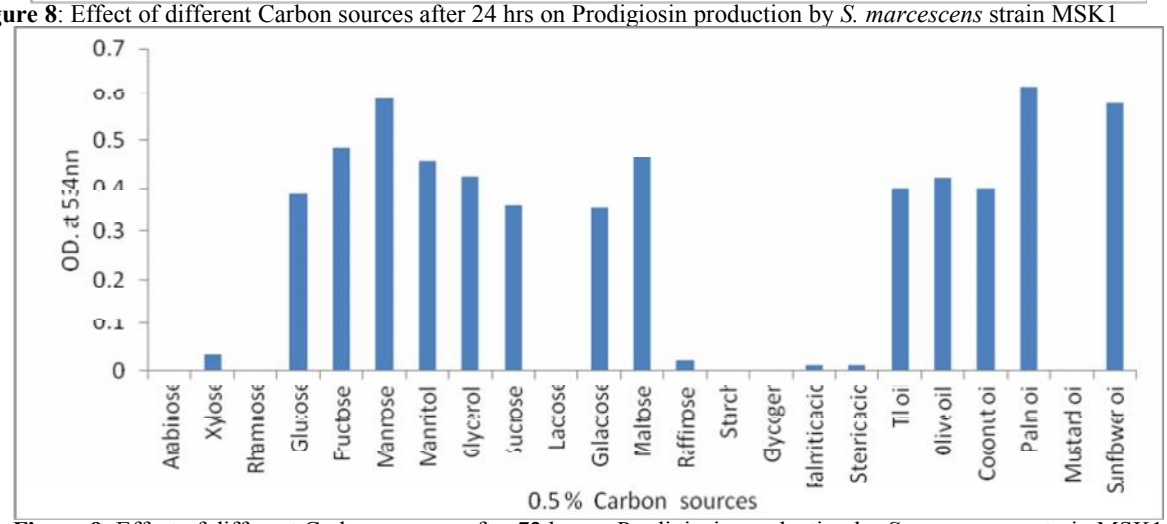

Figure 9: Effect of different Carbon sources after 72 hrs on Prodigiosin production by *S. marcescens* strain MSK1

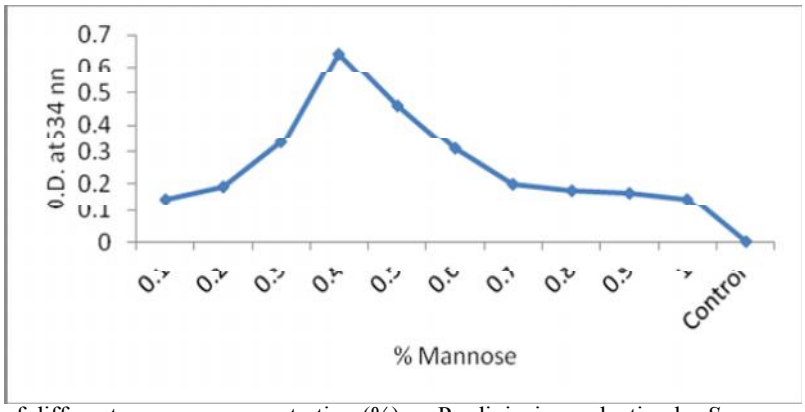

Figure 10: Effect of different mannose concentration (%) on Prodigiosin production by *S. marcescens* strain MSK1

Optimization of Prodigiosin production

Optimization of amino acids

Initially, different range of Cysteine (0.01-0.1%) was studied on Prodigiosin production of which 0.01% supported maximum production of Prodigiosin and showed a diminution in production of Prodigiosin with increase in concentration of Cysteine (Fig. 4) and therefore the range was expanded, from 0.001-0.010% and it was found to be 0.006% (Fig. 5). Optimized Prodigiosin production was at 0.02% Methionine as shown in figure 6. Combination of Methionine (0.01%) and Cysteine (0.003%) exhibited maximum production of Prodigiosin (Fig.7).

Effect of different carbon sources on Prodigiosin production

After 24 hrs, Mannose resulted in maximum Prodigiosin yield as compared to other C sources. On the contrary, in presence of oils and fatty acids, no Prodigiosin was produced (Fig. 8).However, after 72 hrs there was no increase in Prodigiosin yield in the medium containing mannose or any other sugar, but medium containing oils resulted in production of Prodigiosin (Fig. 9). Palm oil proved to be the best oil as a substrate for Prodigiosin production and resulted in almost same amount of Prodigiosin as that by Mannose. Saturated fatty acids, however, didn't result in Prodigiosin production. Ribose (a pentose sugar) has been shown have inhibitory effects on Prodigiosin yield and similarly other pentoses too did not result in Prodigiosin production (Fig. 8 and Fig. 9).

Optimization of mannose concentration

Effect of different concentrations of mannose was studied and it was found that at 0.4% of Mannose resulted in maximum Prodigiosin yield. Higher concentrations of mannose inhibited Prodigiosin production (Fig. 10).

Effect of different nitrogen sources on Prodigiosin production

Among all the N sources, 0.1% Ammonium chloride supported maximum Prodigiosin production (Fig.11). In presence of Ammonium sulphate without Fe, good pigmentation was observed, however, in presence of Ammonium Ferrous sulphate only little Prodigiosin was produced as compared to that with Ammonium sulphate.

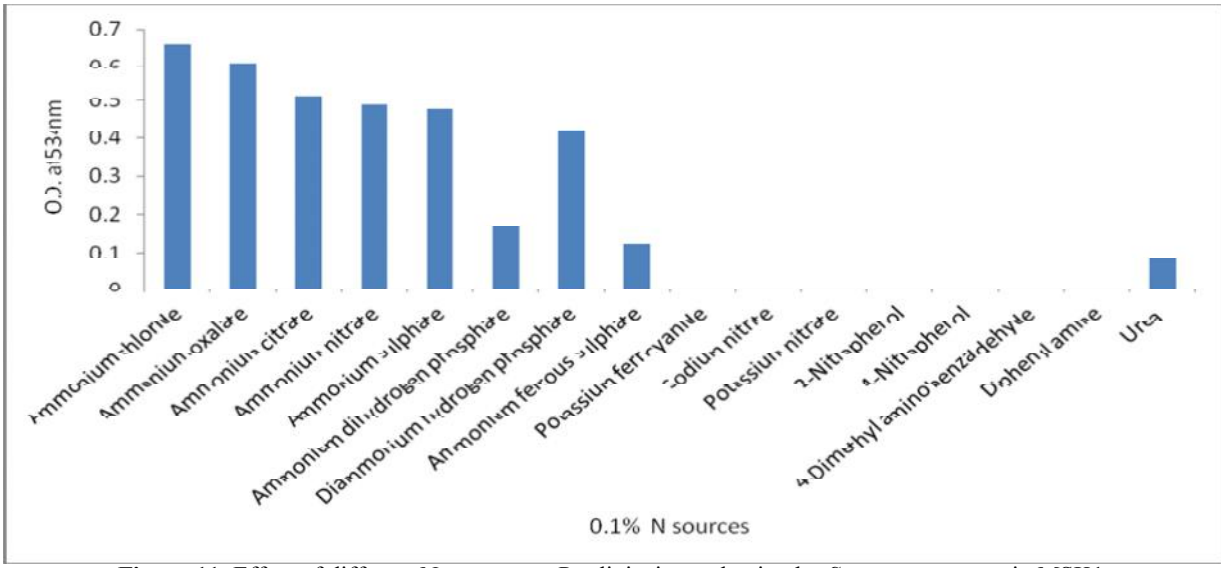

Figure 11: Effect of different N sources on Prodigiosin production by *S. marcescens* strain MSK1

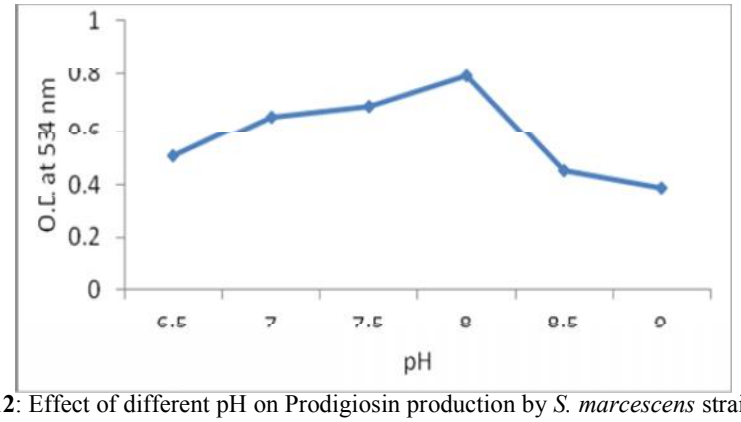

Figure 12: Effect of different pH on Prodigiosin production by *S. marcescens* strain MSK1

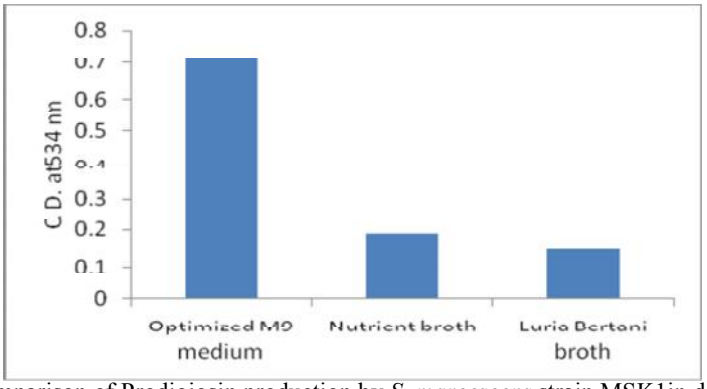

Figure13: Comparison of Prodigiosin production by *S. marcescens* strain MSK1in different media

Figure 14: TLC of Prodigiosin extracted from *S. marcescens* MSK1

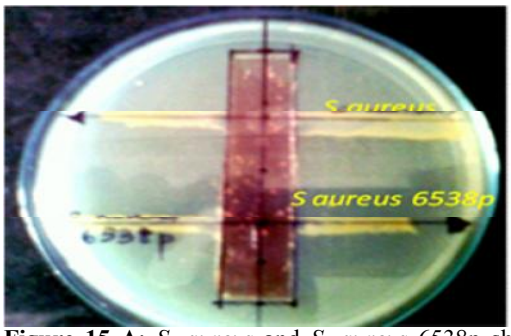

Figure 15 A: *S. aureus* and *S. aureus* 6538p showing no growth on ditch containing Prodigiosin extracted from *S. marcescens* MSK1

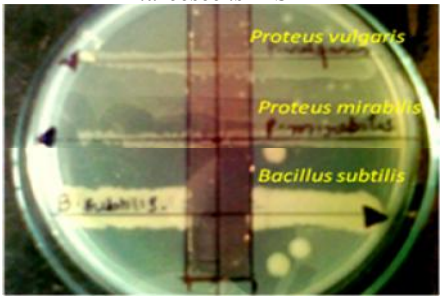

Figure15 B: *P. vulgaris. P. mirabilis* showing growth, whereas *B. subtilis* showing no growth on the ditch containing Prodigiosin extracted from *S. marcescens* MSK1

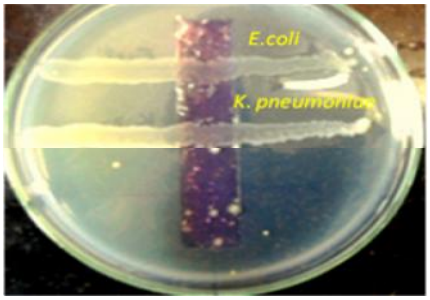

Figure 15 C: *E.coli and K. pneumoniae* showing growth on the ditch containing Prodigiosin extracted from *S. marcescens* MSK1

Effect of pH on Prodigiosin production

The optimum pH for Prodigiosin production by *S. marcescens* MSK1 was 8.0 (Fig. 12).

Comparison of Prodigiosin production in optimized M9 medium with Nutrient broth and Luria Bertani broth

As seen from the results, the optimized M9 medium exihibited far better yield of Prodigiosin as compared to complex Nutrient broth and Luria Bertani media (Fig. 13).

Thin layer chromatography (TLC)

The TLC profile of the Prodigiosin under study showed 3 major bands: purple (Rf 0.1), light orange (Rf 0.2) and dark orange (Rf 0.73) (Fig.14). Dark orange band initially was pink in colour which when exposed to air turned orange.

Anti bacterial effect of Prodigiosin

Antibacterial effect showed that Prodigiosin molecule produced by *S. marcescens* MSK1 strain is effective against gram positive *Staphylococcus aureus, S. aureus 6538p, Bacillus subtilis* strains (Fig.15A, Fig.15B, and Fig.15C) but not against gram negative *Klebsiella pneumonia, Proteus vulgaris and P. mirabilis* strains.

DISCUSSION

A Prodigiosin producing bacterium isolated from air and identified as *S. marcescens*MSK1. Many strains of *Serratia marcescens* were studied for Prodigiosin production include Nima (Qadri and Williams, 1972; Qadri and Williams, 1973), B2 (Someya *et al.,* 2004), Swift-1 (SS-1) (Wei and Chen, 2005) and A3 (2) (Cerdeno *et al.,* 2001). Maximum absorption spectrum of the Prodigiosin produced by *Serratia marcescens* MSK1 was in accordance with the literature (Williams *et al.,* 1955). *Serratia marcescens* MSK1 strain exhibited pigmentation in the presence of the amino acids Cysteine and Methionine individually along with 0.5% glucose. However, with this isolate, pigmentation was found to be absent with Proline and Tryptophan which have pyrrole structures and are known to enhance pigmentation (Wei and Chen, 2005). Nonpigmented bacteria obtained by growth of *Serratia marcescens* at 38^0C synthesized Prodigiosin at 25^0C if certain individual amino acids (DL-histidine, L-proline, L-hydroxyproline, DL-alanine, L-alanine, DL-aspartic acid, D-alanine, DL-proline, L-serine, L-ornithine, L-glutamic acid and D-proline) were added to cultures of nonproliferating cells. DL-methionine was not effective alone but at a low concentration enhanced and accelerated biosynthesis of Prodigiosin takes place in the presence of other suitable amino acids (Qadri and Williams, 1972). In another study with orange mutant (OF) of *S. marcescens*, it was indicated that the methyl group on C6 of Prodigiosin comes from Methionine and hence an important role of methionine in biosynthesis of Prodigiosin is methylation of the pigment (Qadri and Williams, 1973). In addition, isotope from Cysteine and Methionine, was found to be incorporated, to a small extent in Prodigiosin (Qadri *et al.,* 1974). The significance of the results is the fact that pigmentation is seen in presence of Cysteine and Methionine individually than with Proline, Tyrosine and Histidine. However, in *Serratia marcescens* strains amino acids with pyrrole like structures Tryptophan, Proline and Histidine in the presence of yeast extract increased pigmentation and Proline is known to be directly incorporated into the Prodigiosin structure (Wei and Chen, 2005; Lim *et al.*, 1976).

At higher concentrations Methionine and Cysteine results in less yield or none of Prodigiosin by *Serratia marcescens* MSK1. Also Methionine (0.01%) and Cysteine (0.003%) in combination enhances Prodigiosin production showing to have a synergistic effect. Engineering the biosynthesis of novel Prodigiosin can be possible by providing these amino acids since it is produced through divergent pathways and provision of intermediates of one pathway can lead to production of predominantly one desired type of Prodigiosin.

Prodigiosin, a typical secondary metabolite is appearing only in the later stages of bacterial growth (Harris *et al*., 2004). The production of Prodigiosin has been shown to be influenced by numerous environmental factors including media composition and pH ((Weinberg, 1970; Williamson *et al*., 2005). Furthermore, the formation of Prodigiosin by *S. marcescecns* MSK1 in the buffered medium with a pH of 8.0 was 10 times greater than the formation in an unbuffered medium. The pH can affect the two functions of Proline which induced pigmentation in Nima strain of *S. marcescens* in different ways. At pH 8.0 the proline oxidase activity would be minimal and more Proline could be incorporated into Prodigiosin (Sole *et al.,* 1994). Therefore, it could be presumed that a similar effect of pH might be seen on enzymes involved in metabolism of Methionine and Cysteine by *S. marcescens* MSK1.

S. marcescens strains are the major producers of Prodigiosin and the type of carbon source may play a crucial role in the Prodigiosin production (Furstner, 2003; Giri *et al.,* 2004). There is evidence indicating that *S. marcescecns* grow well on synthetic media using various compounds as a single carbon source. 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 and as a consequence, initial comparative work was done using different C sources which included different types of sugars (pentoses, hexoses, oligosaccharides, polysaccharides), different oils and saturated fatty acids. Also, it was found that glucose may inhibit Prodigiosin production due to catabolic repression or by lowering the medium pH (Wei and Chen, 2005)**.** According to Kim *et al*. (1998) oil gave a better yield over the various carbon (not fatty acid containing seeds) sources tested. 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 the pigment yield was more in media containing oils than in saturated fatty acids which are a deviation as compared to the work done by Giri *et al.* (2004)**,** in which saturated fatty acids gave better results. Ribose (a pentose sugar) has been shown to have inhibitory effects on Prodigiosin yield (Lawanson and Sholeye, 1975).In previous study, the addition of maltose or glucose in sesame medium does not significantly enhance the pigment production. In fact the addition of glucose or maltose caused a reduction in Prodigiosin production which could be due to catabolite repression (Giri *et al.,* 2004).Similarly, in our experiment too mannose at higher concentration resulted in decrease in

Prodigiosin production which may be due to catabolite repression.

S. marcescens wild-type 2G1, grown in glucose-$(NH_4)_2SO_4$ -mineral salts medium, required the addition of 0.02 to 0.03 µg of Fe per ml for maximal growth**.** Prodigiosin was produced within the range 0.1 to 2.0 µg of Fe per ml, with no Prodigiosin synthesized at iron concentrations below and above this range (Silverman and Munoz, 1973). However, *S. marcescens* MSK1 doesn't require Fe in Mannose-$(NH_4)_2SO_4$-mineral salts medium. Presence of Fe results in reduction in Prodigiosin production and this can be elicited from the results with Ammonium ferrous sulphate (Fig. 11).

Optimized M9 medium exhibited maximum Prodigiosin yield compared to Nutrient broth and Luria Bertani broth. In nutrient broth the major components are 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 and hence, the concentration of Cysteine and Methionine might have been above than the optimized value required for pigmentation by *S. marcescens* MSK1. Trace elements, minerals and usually some sugar are also present which may result in less Prodigiosin production due to catabolite repression (Giri *et al.,* 2004; Khanafari *et al*., 2006). It has been stated that Inorganic phosphate inhibits Prodigiosin formation in *Serratia marcescens* (Lawanson and Sholeye, 1975)**.** Other studies have also demonstrated that synthesis of Prodigiosin by non-proliferating cells of *Serratia marcescens* is depended to presence of inorganic phosphate (Pi) concentrations. A high elevation of pigment formation was obtained at less than or equal to 0.3 mM and a broader but much lower elevation was obtained at 10 to 250 mM Pi (Witney *et al.,* 1977). However, optimized M9 medium contains 225 mM Na_2HPO_4 (0.32%) and 220 mM KH_2PO_4 (0.3%) resulted in pigmentation which is contradictory to the above results indicating that the *S. marcescens* strain MSK1 is tolerant to high concentrations of inorganic phosphate. It has been previously reported that production of pigment is controlled by *N*-acyl homoserine lactone (*N*-AHL) quorum sensing, and disruption of *pstS* mimicked phosphate limitation and caused concomitant hyper-production of Prodigiosin (Slater *et al*., 2003). Similarly, in *S. marcescens* MSK1 too, the gene *pstS* seems to be mutated which limits phosphate transportation into the cell and hence, resulting in Prodigiosin pigmentation at such high concentrations.

The TLC findings establish that the Prodigiosin described by Wrede and Rothhaas (1934) and Hubbard and Rimington (1950) is not a single substance, and the profile is not exactly the same but was similar to TLC profile of pigment extracted from cultures of *S. marcescens* strain Nima by similar extraction method, in which acetone-extracted Prodigiosin separated into at least four bands, blue at Rf 0.18, red at Rf 0.48 and 0.70, and orange at Rf 0.89. The orange band at Rf 0.89 had only an evanescent orange color which rapidly turns red when exposed to air. A blue band at Rf 0.18 was consistently present and this component had not been reported previously. The red band at Rf 0.48 was always present, but only in very small amounts (Williams *et al.,* 1955). The presence of the purple component at Rf 0.1discovered during this investigation was suggested in previous reports since they had observed a slow-moving purple band in their chromatograms of Prodigiosin and also the blue band was probably the same purple component reported before (Bunting, 1940; Weiss, 1949; Williams *et al.,* 1955). However, in addition to the above bands, certain bands which were not very distinct were observed. This suggest that the structure may be a derivative of Prodigiosin which may be novel, as expected, as Cysteine and Methionine for the first time induced pigmentation. To confirm this, further work needs to be carried to know its exact structure. Pigment fractions vary with time and even with the slightest variation in extraction and production. The fractions probably arise during post processing.

Prodigiosin produced by *S. marcescens* MSK1 inhibits only gram positive strains. However, there is data which indicates that Prodigiosin extracts inhibit the growth of several species (both gram positive and gram negative) of bacteria (Kalesperis, 1975).

CONCLUSION

A red pigment producing bacterium was isolated from air and identified as *S. marcescens* MSK1. It was found to respond to Methionine and Cysteine for Prodigiosin production in the presence of glucose. Prodigiosin production was optimized to obtain maximum yield in M9 medium with 0.4% mannose, 0.01% methionine, 0.003% cysteine and 0.1% Ammonium chloride, pH 8 at 28^0C under shaker conditions (120rpm) for 24 hrs. Methionine and Cysteine together resulted in better Prodigiosin production and found to exhibit a synergistic effect. Mannose and Palm oil almost yielded same amount of Prodigiosin but since, mannose induced pigmentation within 24 hrs so it was selected as a better C source. Saturated fatty acids didn't result in Prodigiosin production but on the contrary oils did which are found to contain more amounts of unsaturated fatty acids.TLC results were similar to that obtained of Pigment extracted from cultures of *S. marcescens* strain. Pigment also exhibited antibacterial activity against gram positive strains.

REFERENCES

Boger, D.L. and Patel, M. (1988) Total synthesis of Prodigiosin, prodigiosene and desmethoxyProdigiosin: Diels-Alder reactions of hetero cyclic azidenes and development of an effective palladium (II)-promoted 2'2'-bipyrrole coupling procedure. J Org Chem, 53, 1405-1415.

Buchanan, R. E. and Gibbons, N. E. (1975) Bergeys Manual of Determinative Bacteriology 8th edition, 326.

Bunting, M. I. (1940) A description of some color variants produced by *Serratia marcescens*, strain 274. J. Bacteriol., 40, 57-68.

Cerdeno, A.M., Bibb, M.J. and Challis, G.L. (2001) Analysis of the prodiginine biosynthesis gene cluster of *Streptomyces coelicolor* A3(2): new mechanisms for chain

initiation and termination in modular multienzymes. Chem Biol, 8, 817-829.

D'Aoust, J.Y.and Gerber, N.N. (1974) Isolation and purification of Prodigiosin from *Vibrio psychroerythrus*. J Bacteriol, 118, 756-757.

Ding, M. and Williams,R. P. (1982) Biosynthesis of Prodigiosin by White Strains of *Serratia marcescens* Isolated from Patients. Journal of Clinical microbiology, 17 (3), 476-480.

Furstner, A. (2003) Chemistry and biology of roseophilin and the Prodigiosin alkaloids: A survey of the last 2500 years. Chem Int Ed Engl, 42, 3582-3603.

Gargallo, D., Loren, J.G., Guinea, J.andVinas, M. (1987) Glucose-6-phosphate dehydrogenase alloenzymes and their relationship to pigmentation in *Serratia marcescens*. Appl Environ Microbiol, 53, 1983-1986.

Gargallo Viola, D. (1989) Enzyme polymosphism, Prodigiosin production and plasmid fingerprints in clinical and naturally occurring isolates of *Serratia marcescens*. J Clin Microbiol, 27, 860-868.

Gerber, N. N. (1975) Prodigiosin-like pigments. CRC Crit Rev Microbiol. 3(4), 469–485.

Giri, A.V., Anandkumar, N., Muthukumaran, G.andPennathur, G. (2004) A novel medium for the enhanced cell growth and production of Prodigiosin from *Serratia marcescens* isolated from soil. BMC Microbiol, 4, 1-10.

Hardjito, L., Huq, A. and Colwell, R.R. (2002) The influence of environmental conditions on the production of pigment by *Serratia marcescens*. Biotechnol Bioprocess Eng, 7, 100-104.

Harris, K.P., Williamson, R., Slater, H., Cox, A., Abbasi, S., Foulds, I., Simonsen, T., Leeper, J., Salmond, P.C.(2004) The *Serratia* gene cluster encoding biosynthesis of the red antibiotic, Prodigiosin, shows species and strain dependent genome context variation. *Microbiol,* 150, 3547-3560.

Heinemann, B., Howard, A. J., and Palocz, H. J. (1970) Influence of Dissolved Oxygen Levels on Production of L-Asparaginase and Prodigiosin by *Serratia marcescens*. Applied Microbiology, 19 (5), 800-804.

Hejazi, A., and Falkiner, F.R. (1997)*Serratia marcescens*. J. Med. Microbiol., 46, 903-912.

Hubbard, R. and Rimington, C. (1950) The biosynthesis of Prodigiosin, the tripyrryl-methene pigment from *Bacillus prodigiosus*(*Serratia marcescens*). Biochem. J. (London), 46, 220-225.

Kalesperis, G.S., Prahlad K.V. and Lynch D.L. (1975) Toxigenic studies with the antibiotic pigments from *Serratia marcescens*. Can J Microbiol. 21, 213-220.

Kataoka, T., Magae, J., Kasamo, K., Yamanishi, H., Endo, A., Yamasaki, M.and Nagai, K. (1992) Effects of Prodigiosin 25-c on cultured cell lines: Its similarity to monovalent polyether ionophores and vacuolar type H+ -ATPase inhibitor. J Antibiot, 45, 1618-1625.

Khanafari, A., Assadi, M. M. and Fakhr F. A. (2006) Review of Prodigiosin, Pigmentation in *Serratia marcescens.*Online Journal of Biological Sciences 6 (1), 1-13.

Kim, C.H., Sung-Ho, K.and Suk-In,K. (1998)Isolation and Characteristics of Prodigiosin like red pigment produced by *Serratia* sp. KH-95. *Kor J Appl Microbiol Biotechnol.* 26(4), 283-289.

Kobayashi, N. and Ichikawa,Y. (1991) Separation of the Prodigiosin localizing crude vesicles which retain the activity of protease andnuclease in *Serratia marcescens*. Microbiol. Immunol., 35, 607-614.

Lim, D. V., Qadri, S. M. H., Nichols, C. and Williams, R. P. (1976) Biosynthesis of Prodigiosin by Non-Proliferating Wild-Type *Serratia marcescens* and Mutants Deficient in Catabolism of Alanine, Histidine, and Proline. Journal of Bacteriology, 129 (1), 124-130.

Lawanson, A.O. and Sholeye, F.O. (1975) Inhibition of Prodigiosin formation in *Serratia marcescens* by adenosine triphosphate. Experientia, 32, 439-440.

Lewis, S.M. and Corpe, W.A. (1964) Prodigiosin producing bacteria from marine sources. Appl Microbiol. 12, 13-17.

Pryce, L. H., Jones,S., Patel,P., Burnham,S., Knights,K., Powell,J. N., and LaForm,A., (2008) Kinetic Analysis of Growth Rate, ATP, and Pigmentation Suggests an Energy-Spilling Function for the Pigment Prodigiosin of *Serratia marcescens.* Journal of Bacteriology, 190 (22), 7453-7463.

Qadri, S. M. H., and Williams, R. P. (1972) Induction of Prodigiosin biosynthesis after shift-down in temperature of nonproliferating cells of *Serratia marcescens*. Appl. Microbiol. 23 (4), 704-709.

Qadri, S. M. H., and Williams, R. P. (1973) Role of Methionine in Biosynthesis of Prodigiosinby *Serratia marcescens.* Journal of Bacteriology, 116 (3), 1191-1198.

Qadri, S. M. Hussain and Williams, Robert P. (1974)Incorporation of amino acid carbon into Prodigiosin synthesized by nonproliferating cells of *Serratia marcescens.* Canadian Journal of Microbiology, 20(4), 461-468.

Samrot, A. V., Chandana, K., Senthilkumar P. and Narendra, K. G (2011) Optimization of Prodigiosin production by *Serratia marcescens* SU-10 and evaluation of its bioactivity. International Research Journal of Biotechnology (ISSN: 2141-5153), 2(5), 128-133.

Silverman, M. P. and Munoz, E. F. (1973). Effect of Iron and Salt on Prodigiosin Synthesis in *Serratia marcescens*. Journal of Bacteriology, 114 (3), p. 999-1006.

Slater, H., Crow, M., Everson, L. and Salmond, G. P. C. (2003) Phosphate availability regulates biosynthesis of two antibiotics, Prodigiosin and carbapenem, in *Serratia* via both quorum-sensing-dependentand -independent pathways. Molecular Microbiology 47(2), 303–320.

Sole M., Rius,N., Francia,A. and Loren, J. G. (1994) The effect of pH on Prodigiosin production by non-proliferating cells of *Serratia marcescens.* Letters in Applied Microbiology19, 341-344.

Someya, N., Nakajima, M., Hamamoto, H., Yamaguchi, I.and Akutsu, K. (2004) Effects of light conditions on Prodigiosin stability in the biocontrol bacterium *Serratia marcescens* strain B2. J Gen Plant Pathol, 70, 367-370.

Songia, S., Mortellaro, A., Taverna, S., Fornasiero, C., Scheiber, E.A., Erba, E., Colotta, F., Mantovani, A., Isetta, A.M.andGolay, J. (1997) Characterization of the new immunosuppressive drug undecylProdigiosin in human lymphocytes. J Immunol, 158, 3987-3995.

Tsuji, R.F., Yamamoto, M., Nakamura, A., Kataoka, T., Magae, J., Nagai, K.and Yamasaki M. (1990)Selective immunosuppression of Prodigiosin 25-C and FK506 in the murine immune system. J Antibiot, 43, 1293-1301.

Tsuji, R. F., Magae, J., Yamashita, M., Nagai, K.and Yamasaki, M. (1992). Immunomodulating properties of Prodigiosin 25-C, an antibiotic which preferentially suppresses induction of cytotoxic T cells. J Antibiot, 45, 1295-1302.

Wasserman, H.H., Sykes,R.J., Peverada, P., Shaw, C.K., Cushley, R.J. and Lipsky, C.R. (1973) Biosynthesis of Prodigiosin. Incorporation patterns of C-labeled alanine, proline, glycine and serine elucidated by fourier transform nuclear magnetic resonance. J. Am. Chem. Soc., 95, 6874-6875.

Wei, Y.H. and Chen, W.C. (2005) Enhanced production of Prodigiosin-like pigment from *Serratia marcescens* SMΔR by medium improvement and oil supplementation strategies. J Biosci Bioeng, 99, 616-622.

Weinberg, E. D. (1970) Biosynthesis of secondary metabolites: roles of trace metals. Advan. Microbial Physiol. 4,1-44.

Weiss, C.M. (1949) Spectrophotometric and chromatographic analyses of the pigment produced by members of the genus Serratia. J Cellular Comp Physiol, 34, 467-492.

Williams, R.P., Green, J.A.andRappoport, D.A. (1955) Studies on pigmentation of *Serratia marcescens*. I. Spectral and paper chromatographic properties of Prodigiosin. J Bacteriol, 71, 115-120.

Williamson, N.R., Simonsen, H.T., Ahmed, R.A., Goldet, G., Slater, H., Woodley, L. and Leeper, F.J., Salmond, P.C. (2005) Biosynthesis of the red antibiotic, Prodigiosin, in *Serratia*: identification of a novel 2-methyl-3-n-amyl-pyrrole (MAP) assemblypathway, definition of the terminal condensing enzyme, and implications for undecylProdigiosin biosynthesis in Streptomyces. Mol Microbiol*,* 56, 971-989.

Witney, F.R., Failia, M.L.and Weinberg, E.D. (1977) Phosphate inhibition of secondary metabolism in *Serratia marcescens*. Appl Environ Microbiol, 33, 1042-1046.

Wrede, F. and Rothhaas, A. (1934)Uber das Prodigiosin, den rotenFarbstoff des *Bacillus prodigiosus*. VI. Hoppe-Seyler's Z. physiol. Chem., 226, 95-107.