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Production of Prodigiosin from *Serratia marcescens* and its antioxidant and anticancer potential

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Abstract

In the present study sediment samples were collected from Pichavaram mangroves during the month of May 2014. The microbial density of the samples was in the range of $1.3x10^4$ to $1.5x10^5$ CFU/g. The most potent strain was identified as *Serratia marcescens*. Regarding optimization, pH-6 was resulted in the maximum biomass (2.6g/L) and prodigiosin production. Likewise, maximum was observed at 25°C and the minimum was at 40°C. 0.4% NaCl concentration was preferred for both biomass and pigment production (2.9g/L and 25 mg/L). 30 hrs of incubation resulted in the maximum production of both biomass and prodigiosin (27.6 g/L and 27.6 mg/L). At static condition 1.2 g/L of biomass and 5.2mg/L of prodigiosin were produced. Both biomass and pigment production was maximum at 150 rpm with 2.0 g/L of biomass and 26.6 mg/L of prodigiosin. 1.5% dextrose was found to be ideal carbon source at which 2.6g/L of biomass and 33mg/L of product was obtained. Yeast extract (1%) as the nitrogen source supported both biomass and pigment production (2.63 g/L and 33 mg/L). 2% inoculum supported the maximum (2.6 g/L biomass and 25.5 mg/L of pigment). 5% peanut oil cake as cheaper substrate resulted in 3.8 g/L of biomass and 40.9 mg/L of product. Biochemical composition of prodigiosin was found to have 68% of protein, 31.2% of carbhohydrates and 0.8% of lipid. The maximum antioxidant activity of 2.4mg/ml of was observed. Regarding cell lines study, the IC_{50} value of HEP2 cell lines was 31.25 µg/ml of prodigiosin concentration.

Keywords: *Serratia marcescens*, Prodigiosin, Pichavaram mangroves, HEP2 cell lines, Pigment.

Introduction

Natural organic pigments are generally extracted from fruits, vegetables, seeds, roots and microorganisms and they are sometimes called biocolours because of their biological origin (Pattnaik *et aI.,* 1997). Natural pigments have been used to replace synthetic dyes in recent decades. Natural pigments have important functions other than the imparted beauty, such as the photosynthesis and probably life all over the world may not be possible without chlorophylls and carotenoids. However, undesirable properties of natural pigments such as solubility and short term stability limit their application in the food industry (Ersus and Yurdagel, 2007).

Although, there are a number of natural pigments, only a few are available in sufficient quantities to be useful for industry because they are usually extracted from plants (Lauro, 1991). In spite of the availability of variety of pigments from fruits and vegetables, there is an ever growing interest in microbial pigments due to several reasons like their natural character and safety to use, production being independent of seasons

and geographical conditions, controllable and predictable yield (Francis, 1987). The rapid growth of microbes reduces the production time to a matter of days. Compared to plant or animal sources, the production is flexible and can easily be controlled (Taylor, 1984).

There are several organisms which can produce pigments, which are one of the important classes of these secondary metabolites and are often referred to as biopigments. These biopigments can be obtained from two major sources, plants (Papageorgiou *et al*., 1979) and microorganisms (Cho *et al*., 2002). Some strains of *Serratia marcescens* produce characteristic red colour pigment called prodigiosin whereas rest does not. The red pigment prodigiosin was isolated from *S. marcescens* way back in 1902 by Kraft (Venil and Lakshmanperumalsamy, 2009).

Characteristics such as antimicrobial, antitumor and antibiotic of prodigiosin make this natural pigment appropriate for medical applications. Besides, due to the unique characteristics of prodigiosin, this pigment is applicable as a colorant in foodstuffs. Consequently, prodigiosin is of interest because of its potential clinical utility. Apart from prodigiosin production *S. marcescens* has many other remarkable applications such as extracellular products including chitinases, proteases, lipases, nucleases, bacteriocins, surfactants and wetting agents (Braun and Schmitz, 1980, Clegg andAllen, 1985, Yanagida *et al*., 1986, Hines *et al*., l., 1988 and Matsuyama *et al*., 1995). The objective of the present study was to find a potential prodigiosin producer from the marine environment.

Materials and Methods

Sample collection

Sediment samples were collected from Pichavaram mangroves using a sterile spatula, transferred aseptically to laboratory and processed immediately for isolation of strain.

Isolation of prodigiosin producing bacteria

Sample was spread plated on modified nutrient agar plates prepared with 50% aged sea water instead of distilled water and incubated at 37^{o}C for 48hrs. Plates were inspected for brick red colored colonies that indicated the production of prodigiosin (Kamble and Hiwarale, 2012).

Screening of potential strain

Screening was done by the formation of a red or pink colour formation in the acidified solution and yellow colour in alkaline solution indicated a positive, presumptive test for prodigiosin (Gerber and Lechevalier, 1976). The potential strain producing maximum prodigiosin was identified with the help of Bergy's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

Optimization of different parameters for maximum prodigiosin production

Cultural conditions like pH (5, 6, 7, 8 and 9), temperature (20^{o}C to 45^{o}C with an interval of 5^{o}C), salinity (0 % to 1 %, with an interval of 0.2%) and inoculum size (1 to 5 %, with an interval of 1% - 1 ml of inoculum of log phase culture contained 6.5 × 10^{7} CFU/ml) and incubation period (0 hr to 48 hrs with an interval of 6 hrs) were examined for maximum production of prodigiosin using the isolated potential strain. Agitation was maintained at a range of 50-200 rpm. Different carbon sources (1%) such as glucose, maltose, fructose, sucrose and starch, different concentration of potent carbon source (glucose) (0.5 – 3%), different nitrogen sources (0.5%) such as peptone, beef extract, gelatin, ammonium nitrate and ammonium sulphate, different concentration of potential nitrogen source (yeast extract) (0.2- 1.6%) were tried.

Experiments were carried out using search technique (i.e) varying one parameter at a time, in 250 ml Erlenmeyer flasks containing nutrient broth experiments were carried out in triplicates and the average values were taken into account. The estimation of prodigiosin throughout the optimization study was done using the spectrophotometric assay at 499nm. All optimization studies were done in modified nutrient broth.

Cheaper sources

Cheaper sources like cassava starch, corn starch, peanut oil cake, sesame oil cake, coconut oil cake were inspected for the maximum production of prodigiosin. 5g each of the cheaper sources was added separately in conical flask containing 100 ml 50% aged sea water. Other optimized physical parameters were used. Growth and prodigiosin production were checked as before in shake flasks.

Mass scale production in shake flasks

Standardized cultural conditions and optimized carbon, nitrogen sources as well as cheaper sources were used for large scale prodigiosin production. The production was carried out in 2L Erlenmeyer's conical flask. After incubation period, the growth in terms of biomass of the bacteria and prodigiosin production were examined as before.

Mass scale production in fermentor

The parameters optimized for shake flasks were used for mass scale production in a 3L lab scale fermentor and incubated at 150rpm agitation for 30 hrs. DO (Dissolved oxygen) was maintained at 60%. Fermentor study was done separately for C and N sources as well as for the ideal cheaper substrate (i.e) 5% peanut oil cake. Growth was estimated spectrophotometrically at 600nm and prodigiosin after required processing estimated at 499 in spectrophotometer.

Extraction of prodigiosin

After the large scale production, the cells were harvested by centrifugation at 3000rpm for 30min. The cell pellet was collected and resuspended in acidified methanol (24:1 ratio of methanol and 1mol/l HCl,v/v), vortexed and centrifuged under the same conditions. The supernatant showed pink colour indicated the presence of prodigiosin and was dried with the help of a rotary evaporator. This powder was used for further study.

Biochemical composition

The extracted crude prodigiosin from the above procedure was characterized for its biochemical compounds present in it. The amount of protein present in prodigiosin was estimated by using Lowry *et al.*, (1951). The total carbohydrate amount of prodigiosin extract was estimated by using phenol-sulphuric acid method (Chaplin and Kennedy, 1994). Total free fatty acid in the prodigiosin was estimated by using the method of Sadasivam and Manickam (2004).

Antioxidant and Anti-proliferative activity

Antioxidant activity of the prodigiosin was performed by Hydrogen peroxide method (Ruch *et al.*, 1984). The total antioxidant assay was performed according to Pourmorad *et al.*, 2006.

Cytotoxic potential of the isolates were tested on Human epidermoid larynx carcinoma cell lines (Hep2 cell lines). Viable cells present in the medium formed crystals which were dissolved by adding solublizing reagent Dimethyl sulphoxide (DMSO) that resulted in formation of purple colour. The absorbance of the suspension was measured spectrophotometrically at 695nm by taking DMSO as a blank. Cell viability (%) = (Mean OD/Control OD) x 100 (Van and Viljoen, 2002).

Results and Discussion

In the present study mangrove sediment samples were collected from Pichavaram mangroves during the month of May 2014 (Fig. 1). Brick red/yellow colored colonies on modified nutrient agar plates were selected. The microbial density of the sample was found to be in the range of $1.3x10^4$ to $1.5x10^5$ CFU/g (Fig- 2a). Screening of the most potential strain for prodigiosin production was based on intense pink color formation on nutrient agar plate the (Fig.2b). The most potential strain was identified by biochemical tests as *Serratia marcescens* using Bergy's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) (Table -1).

Fig. 1: Sampling station (Pitchavaram Mangroves)

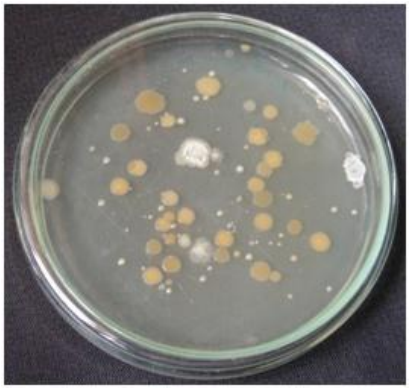

Fig. 2a: Isolation of prodigiosin producing bacteria from marine sediment sample

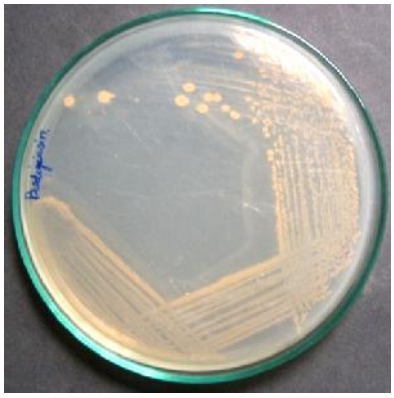

Fig. 2b: Isolated potent prodigiosin producing strain

Table – 1: Biochemical characteristics of *Serratia marcesence*

S.No	Biochemical reactions	Results
1	Gram staining	+
2	Shape	Rod
3	Spore formation	-
4	Motility	+
5	Oxidase	-
6	Catalase	+
7	Indole	-
8	Citrate utilization	-
9	Methyl red	-
Carbohydrate fermentation		
10	Maltose	A+, G-
11	Trihalose	A+, G-
12	Sucrose	A+, G-
13	Fructose	A+, G-
14	Lactose	A+, G-
15	Galactose	A-, G-
16	Mannose	A-, G-
17	Rhamnose	A-, G-
18	Arabinose	A-, G-
19	Raffinose	A-, G-
20	Sorbitol	A+, G-

21	Inositol	A-, G-
22	Xylose	A+, G-
23	Melibiose	A+, G-
	Amino acids	
24	Lysine	-
25	Serine	+
26	Cysteine	-
27	Asparagine	+
28	Ornithine	+
29	Threonine	+

In the present study pH-6 was optimum which resulted in the maximum biomass (2.6g/L) and maximum prodigiosin production. When the temperature range of 20°C – 40°C was examined biomass obtained was from 1.49g/L to 2.3 g/L where as prodigiosin was in the range of 9 mg/L to 25.6 g/L. The maximum was observed at 25°C and the minimum was at 40°C (Fig. 4). In the present study 25°C was found to be suitable for the maximum prodigiosin production at which 256 mg/L was produced. Nilam and Chincholkar (2014) found 28°C as ideal. Maximum yield of prodigiosin from S. *marcescens* was observed at 28°C, while at 37°C no pigment production was observed and the culture broth was white in colour (Giri *et al.*, 2004).

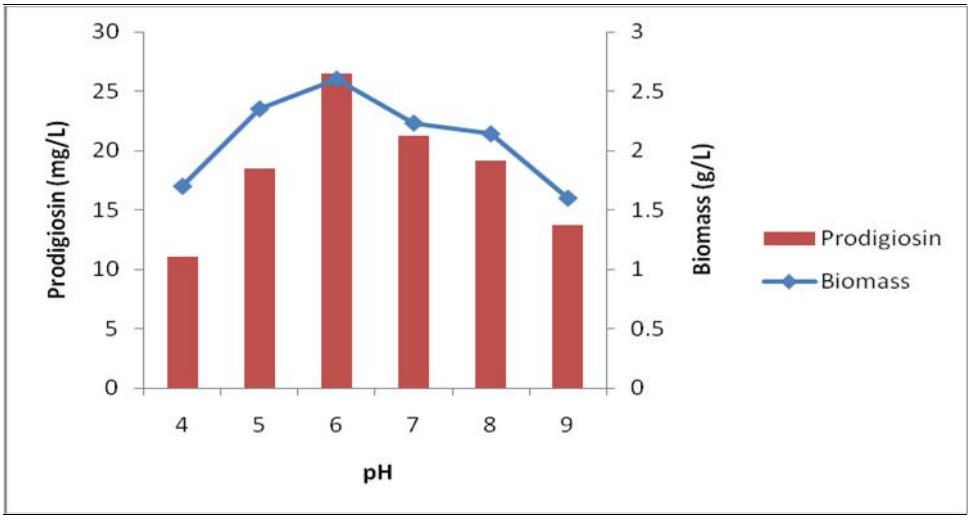

Fig - 3: Effect of pH on growth and production of prodigiosin

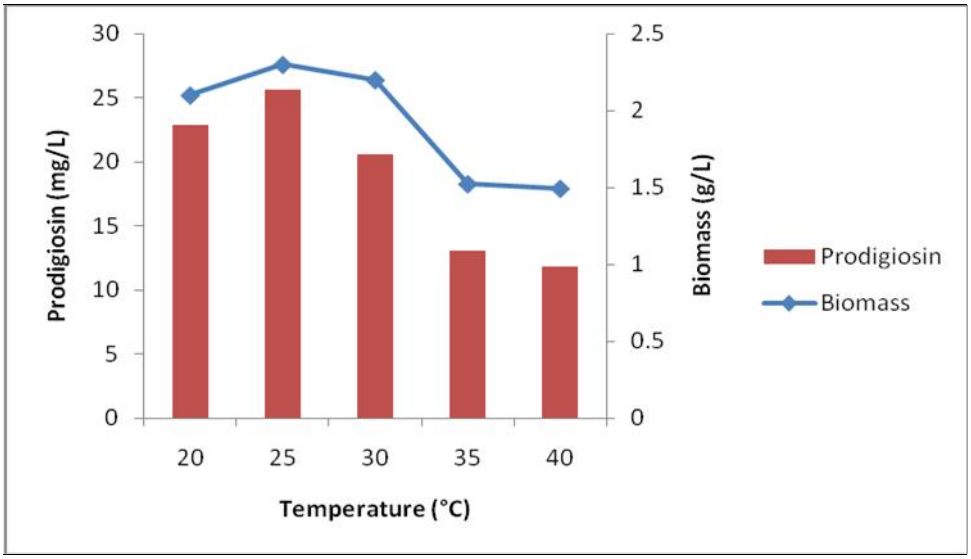

Fig - 4: Effect of temperature on growth and production of prodigiosin

Regarding the NaCl concentration in the present study, 0.4% was found to be optimum for both biomass and pigment production (Respectively 2.9g/L and 25 mg/L) (Fig. 5).In a work done by Krishna (2008), from 10mM onwards increasing trend was noted and maximal pigment and biomass were recorded with 200mM sodium chloride. *Serratia* sp. BTWJ8 used by them can be considered as a slightly halophilic form since it tolerated NaCl concentration from 10mM to 1000mM (0.05 to 5.8 %). However the *S. marcescens* strain used in the present investigation was a non halophilic form, as beyond 0.4% NaCl, both growth and pigment production decreased.

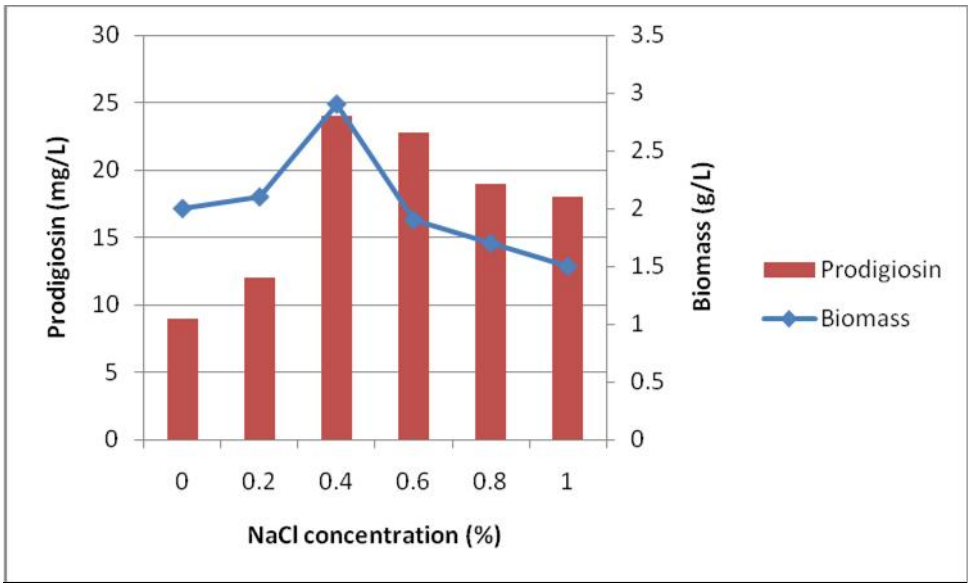

Fig - 5: Effect of NaCl concentration on growth and production of prodigiosin

In the present work, 30 hrs of incubation resulted in the maximum production of both biomass and prodigiosin (27.6 g/L and 27.6 mg/L) (Fig. 6). Bharmal *et al*., 2012 also reported the same in *S.marcescens* MSK1 strain.

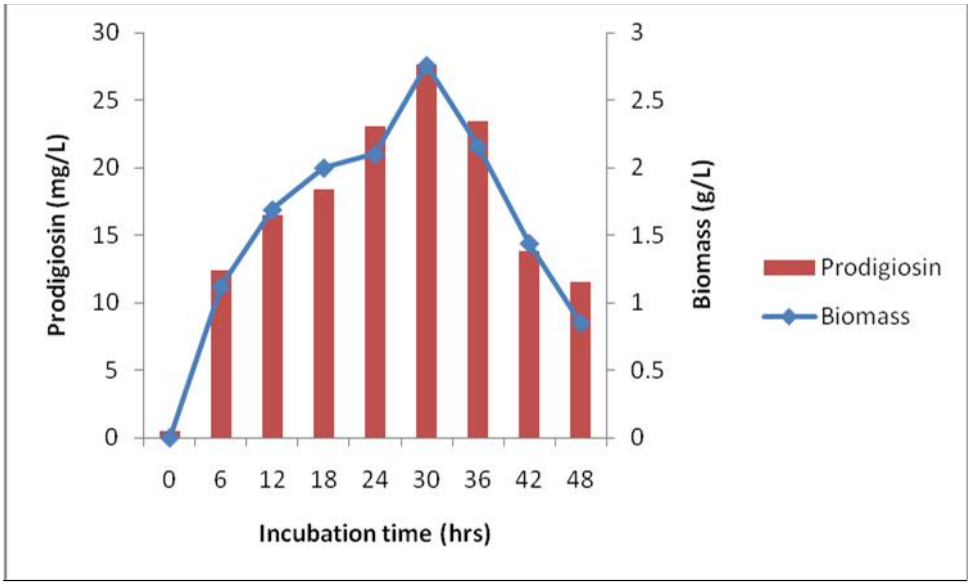

Fig - 6: Effect of incubation time on growth and production of prodigiosin

In the present study both static and agitated conditions were examined. At static condition 1.2 g/L of biomass and 5.2mg/L of prodigiosin were produced. Maximum biomass and pigment production were observed at 150 rpm with 2.0 g/L of biomass and 26.6 mg/L of prodigiosin (Fig. 7). Krishna (2008) also observed 150 rpm as the most ideal.

Regarding carbon source, both biomass and pigment production were found to be maximum in dextrose (2.9 g/L and 27.8 mg/L). (Fig.8). The ideal carbon source dextrose, at 1.5% concentration resulting in the maximum biomass and pigment production as 2.6g/L and 33mg/L respectively (Fig-9). In another study 30mM of dextrose supported the maximum (Krishna, 2008). The present study clearly indicated that the sugars used supported the cell growth, there by prodigiosin production (i.e) if it is a good supporter of growth prodigiosin level was also appreciable. Bharmal *et al*., 2012 found 0.4% of as ideal carbon source for *S.marcescens* MSK 1 strain. Compared to their study, the strains used in the present investigation needed a higher concentration of carbon source.

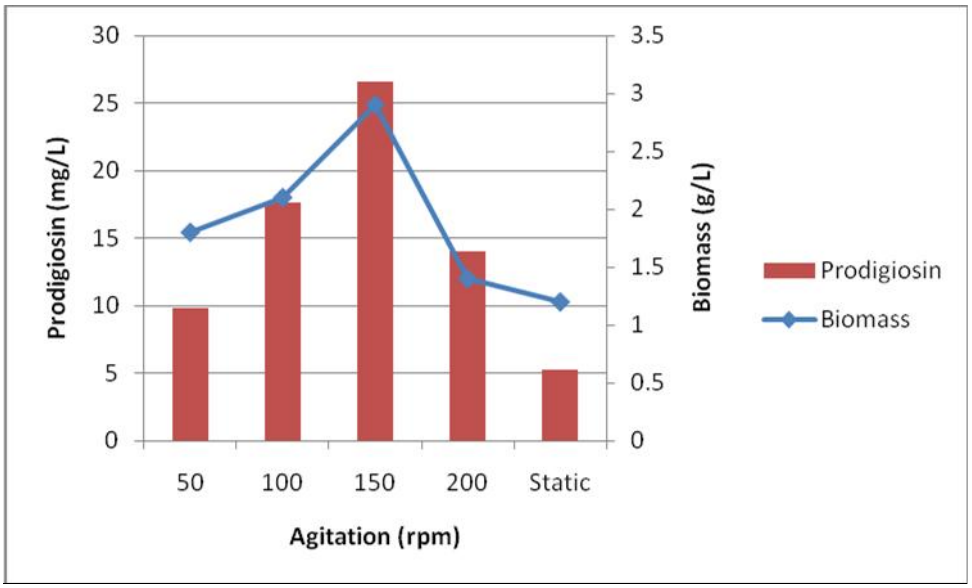

Fig - 7: Effect of agitation on growth and production of prodigiosin

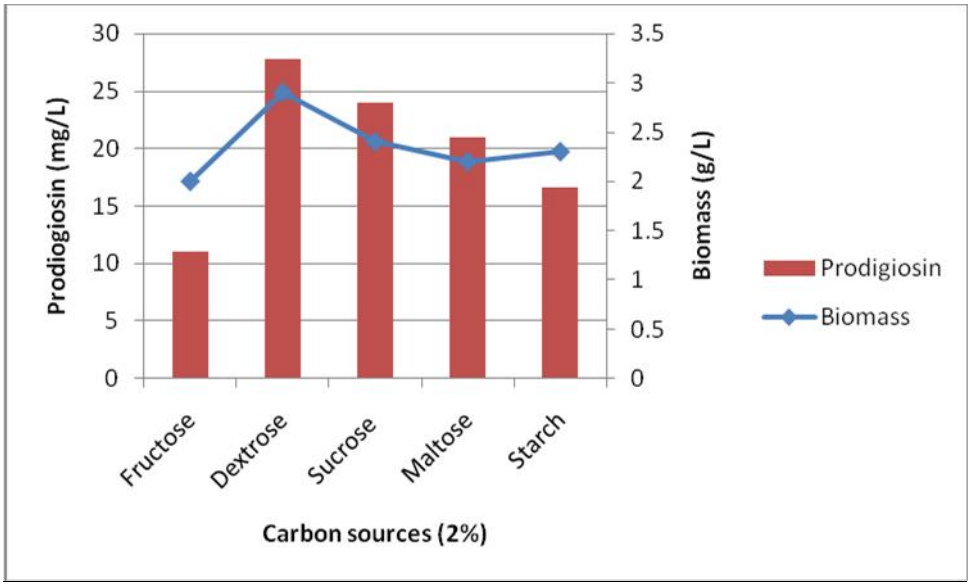

Fig - 8: Effect of carbon source on growth and production of prodigiosin

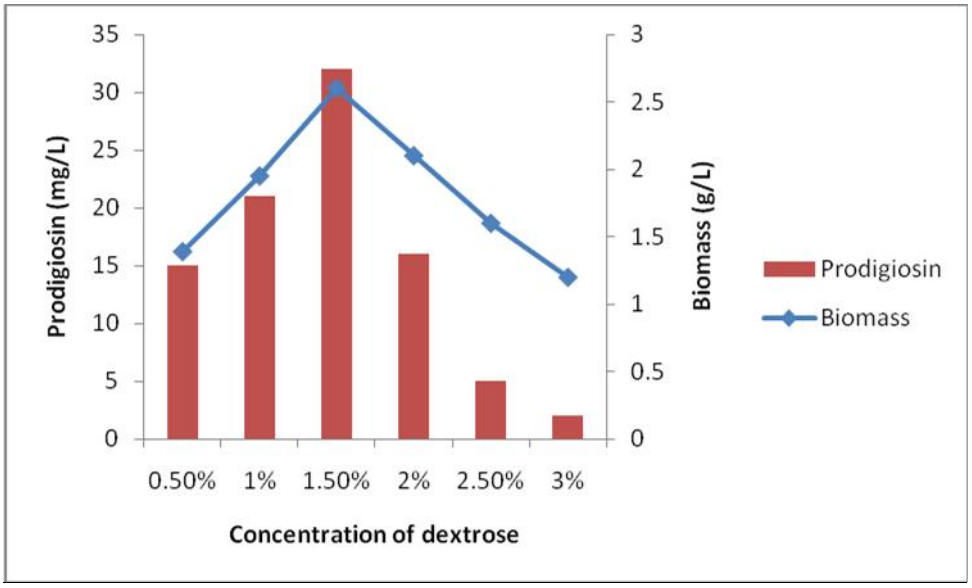

Fig - 9: Effect of ideal carbon source concentration on growth and production of prodigiosin

In the present study, both organic and inorganic nitrogen sources were examined in which yeast extract supported the growth and pigment production resulting in the maximum biomass of 3.4g/L and 31 mg/L of prodigiosin. (Fig. 10). A stimulatory effect of nitrogen source on pigment formation had been reported by Hamdi *et al.*, 1997. As in the present study, among the organic nitrogen sources evaluated, yeast extract enhanced maximal pigment production by *Serratia* sp. BTWJ8 followed by beef extract and malt extract. Regarding the concentration of ideal nitrogen source (i.e.) 1% yeast extract favored the biomass and production of pigment (2.63 (g/L) and 33 (mg/L) respectively (Fig-11).

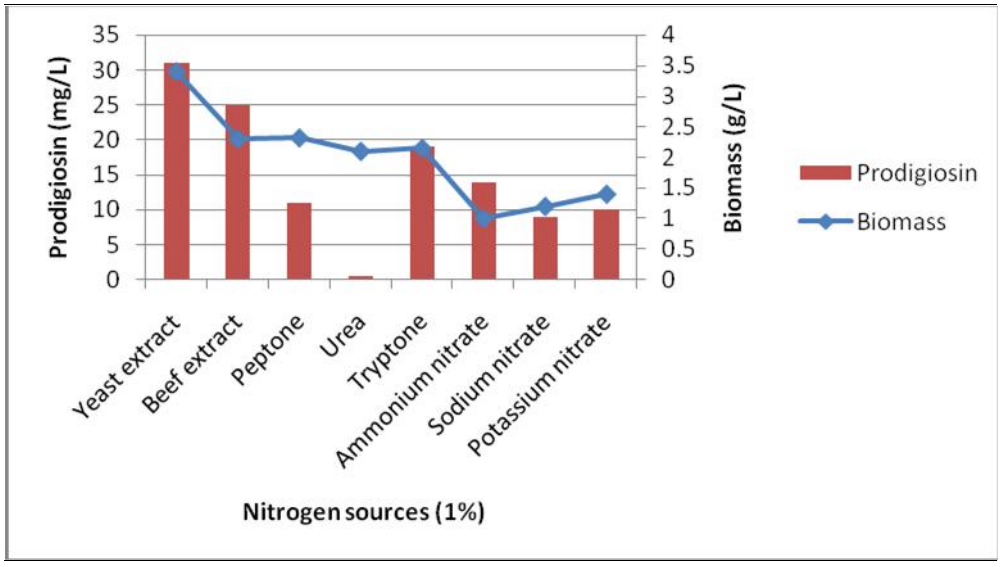

Fig - 10: Effect of nitrogen source on growth and production of prodigiosin

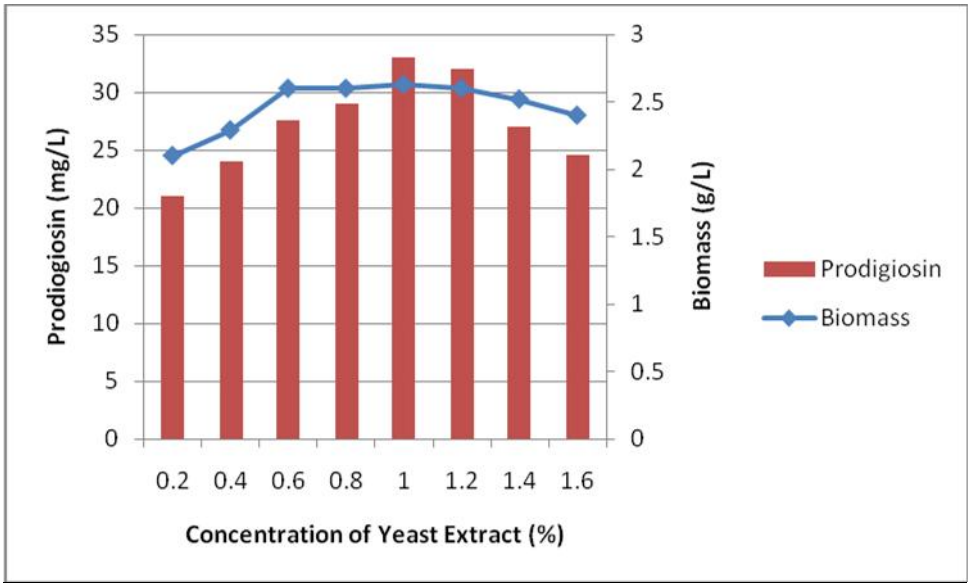

Fig - 11: Effect of ideal nitrogen source (yeast extract) concentration on growth and production of prodigiosin

Inoculum with log phase culture is another important criterion influencing initial growth and production of target products. In the present study also, it was clearly evident, as when 1% to 5% was tried, 2% represented the maximum (2.6 g/L biomass and 25.5 mg/L of pigment) (Fig. 12). Further increase of inoculum resulted in reduction of both biomass and product showing the minimum at 5% concentration. Krishna (2008) using Plackett – Burman design found 1.64 % v/v of inoculum as ideal. Pradeep *et al.*, 2013 found the maximum production of prodigiosin at 5% inoculum.

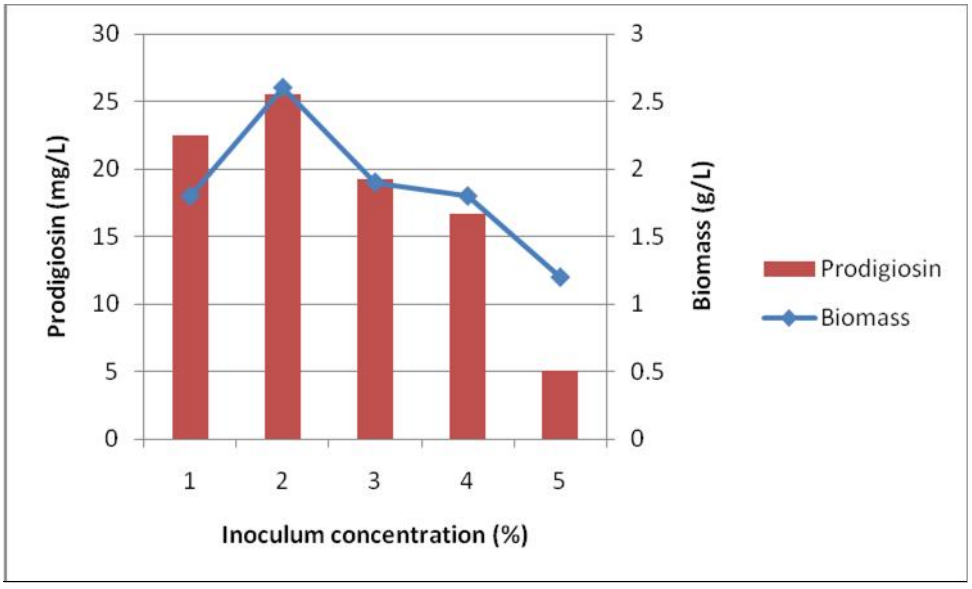

Fig - 12: Effect of inoculum concentration on growth and production of prodigiosin

In the optimized medium, when the carbon (1.5%) and nitrogen sources (1%) were replaced with cheaper substrates (5%peanut oil cake), the maximum biomass and pigment were obtained (3.8 g/L and 40.9 mg/L (Fig. 13). Mass scale production of prodigiosin was carried out in both shake flask and in fermentror using optimized parameters and ideal carbon and nitrogen sources. In the present study a biomass of 3.9g/L and 4.4g/L was obtained in shake flask and fermentor respectively whereas prodigiosin production was observed at a respective concentration of 33mg/L and 36mg/L (Fig. 14 and 15).

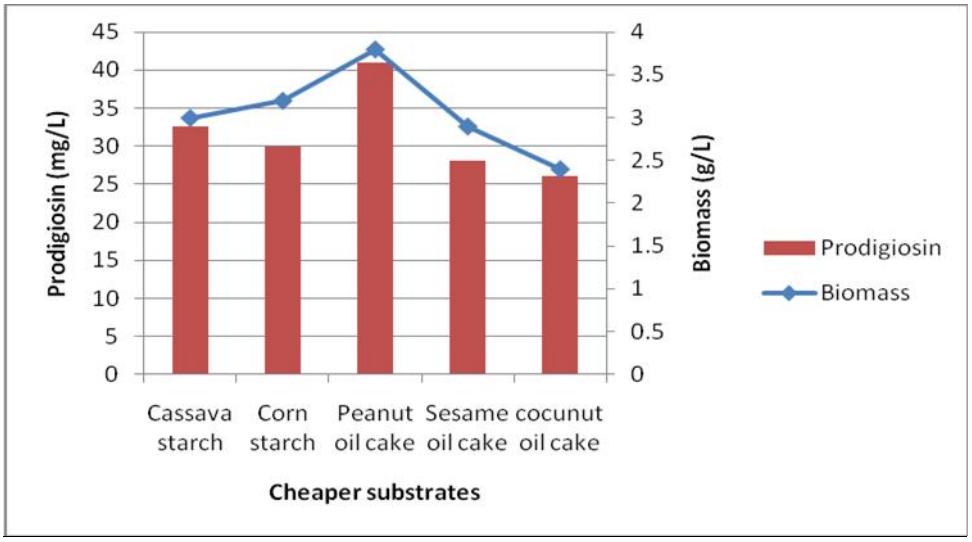

Fig - 13: Effect of cheaper substrates on growth and production of prodigiosin

Fig. 14: Production of prodigiosin in shake flask at 30hrs incubation

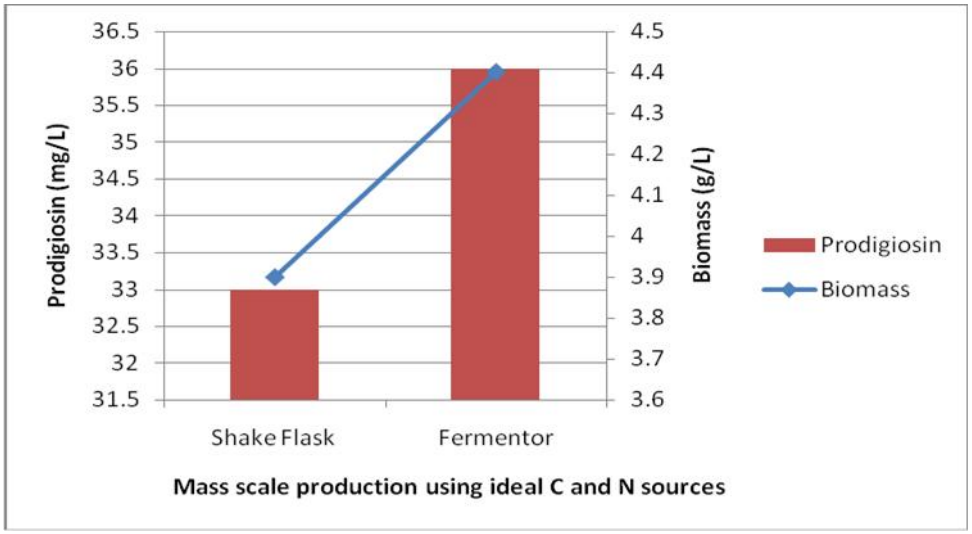

Fig - 15: Mass scale production using optimized parameters and ideal C and N sources at 30hrs incubation

Mass scale production using optimized parameters and ideal cheaper substrate (1% peanut oil cake) resulted in biomass of 3.8g/L and 4.9g/L was obtained in shake flask and fermentor respectively whereas prodigiosin production was observed at a concentration of 40.9mg/L and 50mg/L respectively in shake flask and fermentor (Fig. 16). Pradeep *et al*., 2013 also found peanut powder as the best substrate for the production of prodigiosin. They also tried glucose and maltose at which comparatively lesser production was noted.

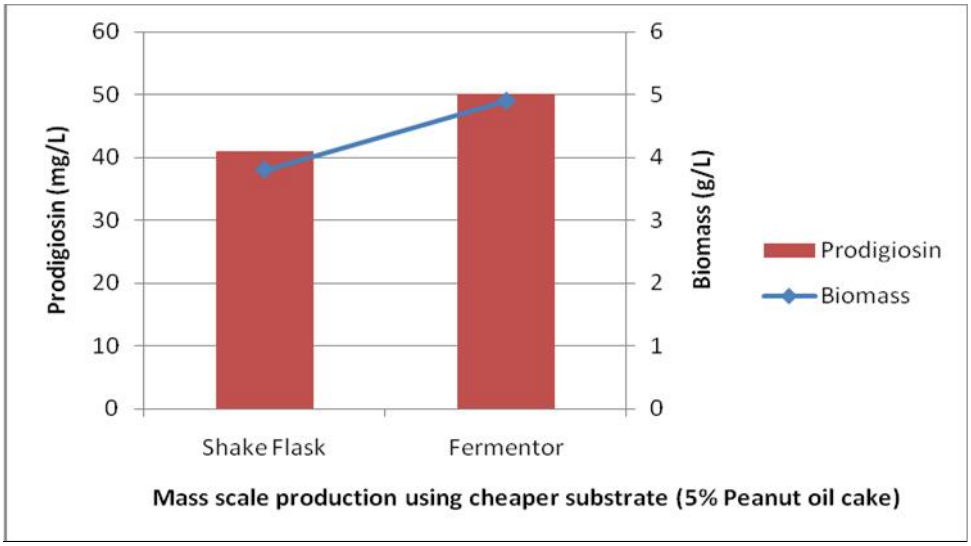

Fig - 16: Mass scale production using optimized parameters and cheaper substrate at 30hrs incubation

In the present investigation when the biochemical estimation or extracted prodigiosin was done using standard procedures it was found to have 68% of protein, 31.2% of carbhohydrates and 0.8% of lipid (Table – 2). Xu *et al.*, 2011 found that the prodigiosin extracted by them was of 60% protein, 38% of carbhohydrate and 1.386% of lipid content. The variation in biochemical content is expected as variations in this pigment were already reported by many.

In the Total antioxidant activity of different concentrations of prodigiosin was assessed. The result showed that the maximum activity of 2.4mg/ml was obtained. H_2O_2 scavenging activity, a maximum rate of 84% was observed (Fig. 17 and 18). Gulani *et al.*, 2012 estimated the total antioxidant capacity of prodigiosin as 22.05 µg AE/mL. Compared to this study prodigiosin obtained from the marine strain seemed to be a better antioxidant.

Table – 2: Biochemical composition

Analysis	Biochemical composition (%)
Protein	68
Carbohydrate	31.2
Lipid	0.8

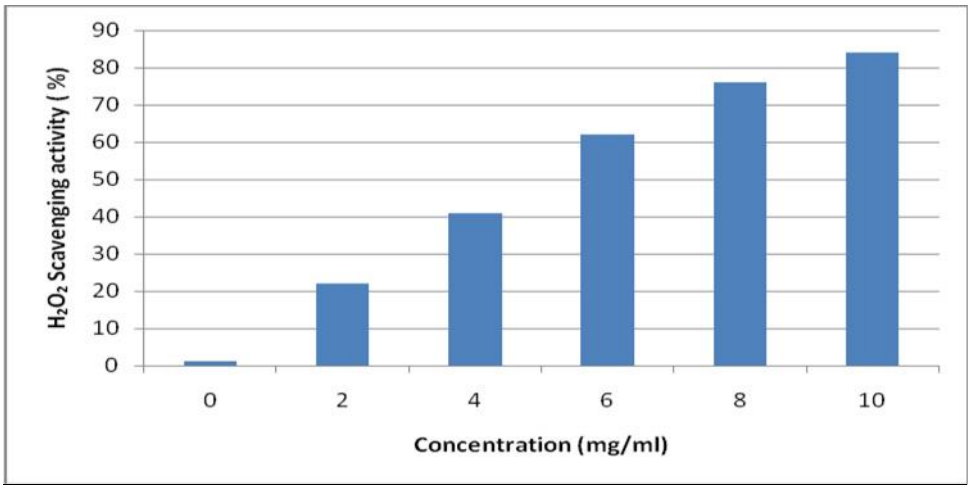

Fig – 17: H_2O_2 scavenging activity of prodigiosin

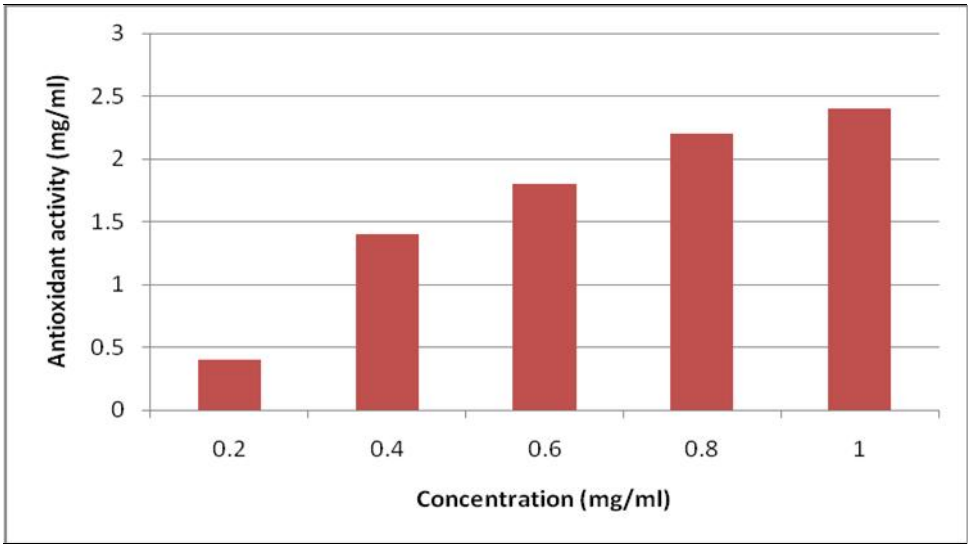

Fig – 18: Total Antioxidant activity of prodigiosin

The prodigiosin exhibited significant inhibition of cell proliferation of HEP_2 cell lines. The highest concentration (1000 µg/ml) the cell viability was in the range of 6.32%. IC_{50} value of HEP_2 cell lines was 31.25 µg/ml of prodigiosin concentration (Fig. 19a and b). Zhang *et al*., 2005 through animal experiment proved that intraperitoneal administration of 5mg kg^{-1} prodigiosin decreased the number of metastatic nodules by 53% and elevated the survival rate of mice about one-fold compared to control group. Khanafari *et al*., 2006 also reviewed on role on prodigiosin on apoptosis. Diaz– Ruir *et al*., 2001 confirmed the apoptotic action of this compound on gastric carcinoma cell lines (HGI-1).

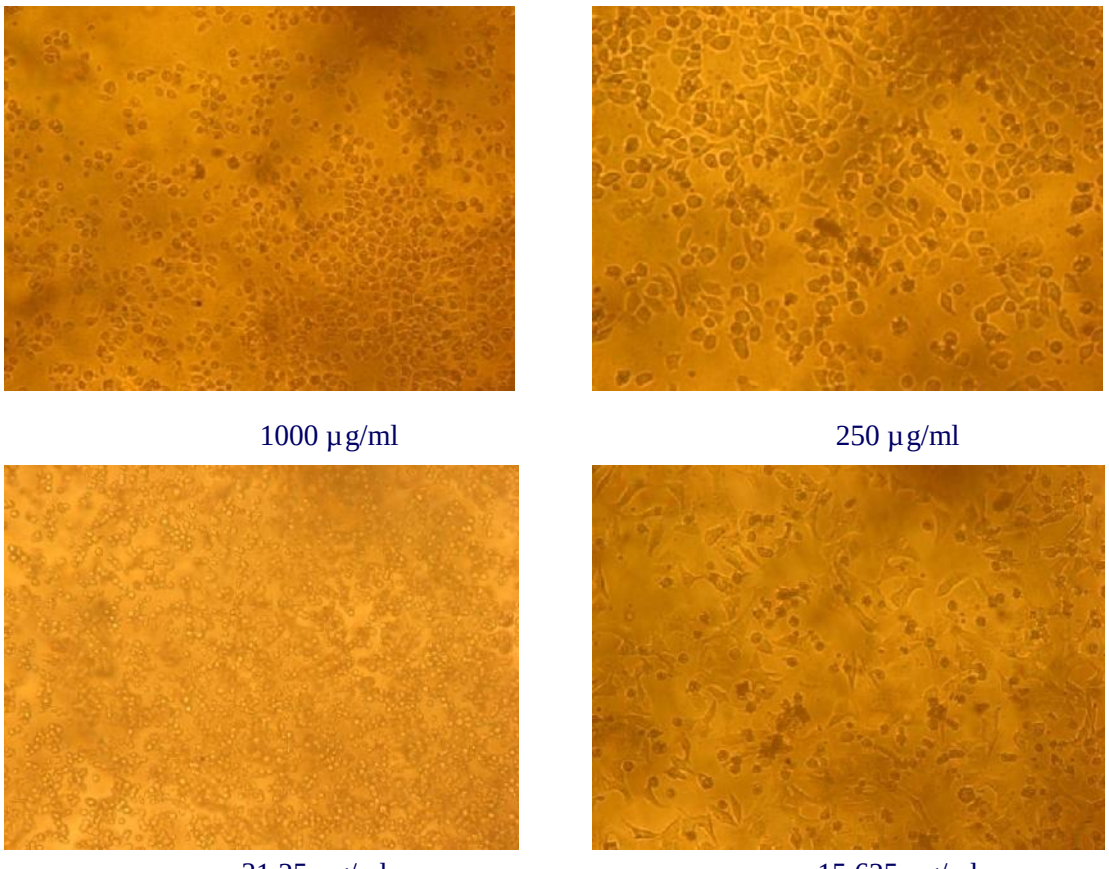

Fig – 19a: Anticancer activity prodigiosin isolated from *S. marcescens* on HEP2 cell lines

S.no	Concentration (µg/ml)	Dilutions	Cell viability
1	1000	Neat	6.32
2	500	1:1	15.55
3	250	1:2	21.03
4	125	1:4	25.56
5	62.5	1:8	35.89
6	31.25	1:16	52.15
7	15.625	1:32	55.63
8	7.8125	1:64	84.12
8	Cell control	-	100

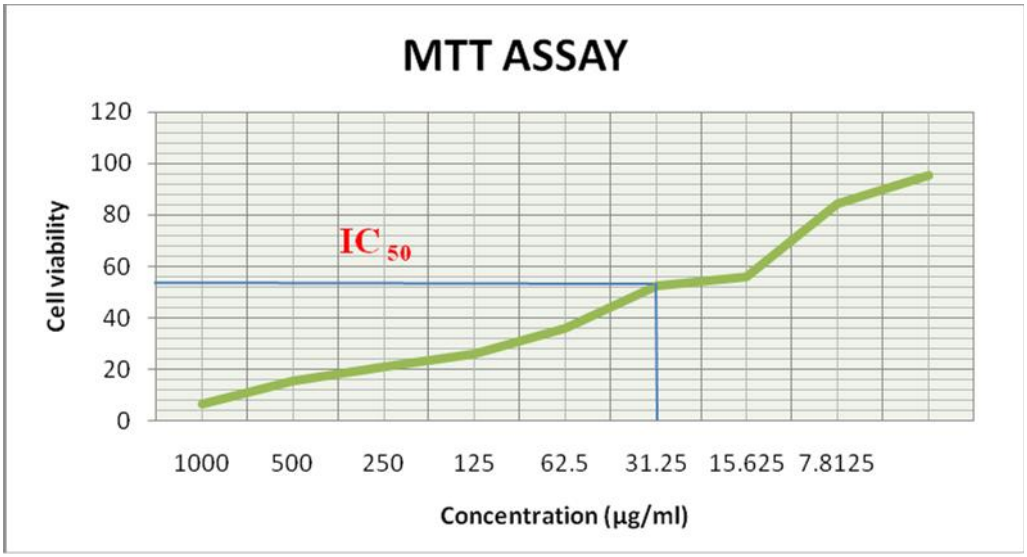

Fig – 19b: Anticancer activity prodigiosin isolated from *S. marcescens*

Conclusion

The *S. marcescens* used in the present study seemed to be a potential producer as for the concentration produced and process parameters preferred. Antioxidant and anticancer potential of the isolated prodigiosin enhanced its applied value.

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