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ENHANCED PRODUCTION OF PRODIGIOSIN FROM *Serratia marcescens* UCP 1549 USING VARIOUS ENVIROMENTAL CONDITIONS.

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ABSTRACT

Strain of *Serratia marcescens* UCP 1549 isolated from a semi-arid soil of the Federal University Technology Minna, Nigeria produced the red pigment prodigiosin. Prodigiosin is a promising drug due to its reported antifungal, immuno suppressive and anti-proliferative activities. The objective of this work was to identify a suitable medium to simultaneously enhance *S. marcescens* growth and pigment produced using different composed substrate *S. marcescens* produced the highest level of prodigiosin (25.10 mg/ml) and (8.43 mg/ml) at 72 hours of cultivation using 50 ml peanut seed broth and sesame seed broth with maltose (0.5%) at 25^{0}C Neutral pH was observed to give the optimal pigment and biomass production (25.10 µg/l) and (2.4 g/l).Media with high acidic (pH 2.0-4.0) and high alkaline (pH 10.0-13.0) did not support pigment and biomass production. Both pigment and biomass showed a direct relationship, both in their increase as well as decrease, in response to variations in pH agitation was at 150 rpm. Fatty acid and maltose play a role in the enhanced cell growth and prodigiosin production. The purified pigment extracted from the biomass when analyzed by mass spectrophotometry showed the expected molecular weight of 321Da corresponding to prodigiosin. This study has successfully shown that, peanut seed broth at temperature of 25^{0}C, pH 7 and 150 rpm was found to be optimal in enhancing production of high yield of prodigiosin.

Keywords: Prodigiosin; Bacteria, growth media, *Serratia marcescens*, pH.

INTRODUCTION

Prodigiosin (5[C3-methoxy-5pyrrol-2-ylidene-pyrrol-2-ylidene-methyl]-2-methyl-3-pentyl-lH-pyrrole) is a secondary metabolite with a unique tripyrrole chemical structure. It is a red pigment isolated from a few species such as *Serratia, Pseudomonas* and *Streptomyce*s (Giri, *et a*l., 2004; Song *et al.,* 2006). It has three rings forming a pyrrolpyromethane skeleton with a C-4 methoxy group, a molecular formula $C_20H_{25}N_3O$ and molecular weight of 323.44 Da (Harris *et al*., 2004 Song *et al.,* 2006). It is sensitive to light and insoluble in water. It is moderately soluble in alcohol and ether, and soluble in chloroform, methanol acetonitrile and DMSO (Grimout*et al*., 1977; Khanafari*et al*., 2006)

Serratia marcescens is a rod-shaped,gram negative, facultative bacterium belonging to the enterobacteriaceae family and characterized by its ability to produce the red pigment prodigiosin (Khanafari *et al.,* 2006). Chromogenic species are usually isolated from the environment from water, soil, plants or insects. Ten species of *Serraria* have been described, of which only three are capable of producing prodigiosin (Grimont *et al*., 1977; Wei *et al*., 2005). The *S. marcescens*is a facultative microorganism and therefore the pigment is produced under aerobic and anaerobic conditions (Wei *et al*.,2005). However, pigmentation is only present in a small percentage of isolated cultures among different. *S. marcescens* strain and depend on many factors such as species type, incubation time, pH, carbon and nitrogen source and inorganic salts (Pandey*et al*., 2009; Kim *et al.,* 2009). Some of the strains *S. marcescens* that are not the chromogenic ones constitute a real threat in hospitals (Araujo *et al*., 2009; Barros *et al*., 2005).

Although, prodigiosin has no known defined role in the physiology of the strains in which it is produced, it has antifungal, antibacterial and protozoal activities,, and thus may have potential chemical utility (Moraes *et al*., 2009). Both prodigiosin and its synthetic derivatives have potent and specific immunosuppressive activity, with new targets that are clearly distinct from those of other drugs (D Alessio *et al.,* 2000, Moraes *et al.,* 2009). No doubt, pigment production by *S. marcscens,* will continue to be intrigued to microbiologist, clinician and bioprocess engineer (Carvalho *et al*., 2005; Nakamura *et al.,* 1981). Prodigiosin has been considered effective as a biological control agent against harmful algae in natural water environment (Venil*et al.,* 2009).

Many types of differential and selective media has been used for growth and prodigiosin production however, regular prodigiosin production has been carried out in nutrient broths containing sesame seeds, maltose broth, peptone glycerol broth Giri *et al.* (2004) and a patented medium Nakamura and Kitamura (1981) containing 2% sodium oleate and also oleic acid substitution instead of sodium oleate and triolein alone as substrate and produced a yield of 0.69 mg/ml prodigiosin. Giri *et al.* (2004) had earlier reported the use of powdered sesame seed in water, nutrient broth and peptone glycerol broth as a growth medium for .*S. marcescens*. After having observed sesame seed to give a better yield in terms of prodigiosin biosynthesis further comparison was done with readily available cheaper and cost effective sources like peanut and coconut (Andrade *et al*., 2005). The aim of this work therefore, was to compare simple and cheap nutritional medium, suitable temperature, pH range and effect of agitation to permit the growth and enhanced prodigiosin production by *S. marcescens.*

MATERIALS AND METHODS

Sample collection

Soil sample for isolation of pigment producing *Serratia marcenscens* was collected from Bosso Campus of the Federal University of Technology, Minna, Nigeria in polythene bags and transported to the laboratory for analysis.

Preparation of Media

All Culture media was prepared according to standard methods described by the manufacturers or according to methods described by Cheesebrough (2003).

Experimental Composition and preparation of media used for growth and prodigiosin production.

Nutrient broth (peptone 10 g/l, sodium chloride 5 g/l, yeast extract 3 g/l), nutrient broth with 0.5% maltose, nutrient broth with 0.5% glucose, peptone glycerol broth (meat extract 10 g/l, peptone 10 g/l, glycerol 10%), 2% powdered sesame seed in distilled water, 2% powdered sesame seed with 0.5% maltose in distilled water, 2%

powdered sesame seed with 0.5% glucose in distilled water, 2% powdered peanut seed in distilled water ,and 2% peanut seed broth in distilled water, were the different media used for the production of pigment from the *Serratia marcescens* isolated. 50 ml of each of the broth was prepared in 250 ml glass conical flasks. The pH of all the above media were maintained at 7.0. The various media were sterilized by autoclaving at 120°C for 20 min. Maltose and glucose were filter sterilized and added to the respective media. Peanut and sesame obtained from provisional stores were crushed in a mixer and then sieved to fine particles before preparing the broth. The experiment was conducted in triplicates.

Isolation of *Serratia* strains from soil sample

Ten grams of the soil sample was weighed into 9ml of sterile distilled water and serially diluted and plated on nutrient agar which was fortified with 1% starch. The plates were incubated at 37^0C for 24 h-48 h. Red pigmented colonies were picked and sub-cultured several times on fresh nutrient agar to obtain pure culture. (Fawole and Oso 1998; Cheesebrough, 2003).The pure cultures were maintained on agar slants in the refrigerator (8^0C) for further characterization and identification.

Genetic Characterization of Isolates

Confirmation of identity as *Serratia marcescens* was achieved using 16S rRN Aanalysis i.e., by amplifyingthe 16S rDNA gene by the polymerase chain reaction (PCR) employing two universal primers, 27f and 1525r (Chung, 1985). The PCR product was analyzed by horizontalgel electrophoresis. The 16S rDNA was isolated and extracted, using an Ultra free-MC filter unit (0.45 µm; Millipore Co, Bedford, USA), following manufacturer's instructions. Ligation of the PCR-amplified 16S rDNA into the pGEM-T vector was done following standard procedures. The cloned 16S rDNA was sequenced using aPRISM™ DyeDeoxy Terminator cycle sequencing kit (Applied Biosystem, California, USA) and an Applied Biosystems atomated

Determination of Pigment and Cell Concentration

The cell concentration was monitored spectrophotometrically by measuring Optical Density (OD) at 600 nm and the dry weight of the cells. The dry weight cell concentration (g/L) was found to be 0.8 × $OD_{600nm.}$ The cell-bound pigment was extracted using a modified method of (William *et al.*, 2004). The cell-bound and extracellular pigment was measured spectrophotometrically at 535 nm, and the total pigment was calculated by adding the cell-bound and extracellular values. A 5 mL broth was centrifuged at 10,000 rpm for 5 min. The extracellular pigment was determined directly from cell-free supernatant at pH 7 by adding hydrochloric acid, while the cell-bound pigment was extracted by adding 5 ml ethanol-chloroform (2:18). A typical spectrophotometric scan of the pigment is presented in figure 1

Extraction and Purification of the Pigmented Metabolite (Prodigiosin)

Extraction was carried out according to (Heinemann *et al*., 1970) Prodigiosin was visualized as a fluorescence spot under UV light with Rf value of 0.9 to 0.95. The pigment was purified by column chromatography using silica gel (mesh size 80–100) as the solid matrix for separation of the non coloured impurity from the pigment (Montarner*et al*., 2002)

Growth of *Serratia marcescens* in different media at 25^0C,30^0C and 35^0C.

Fifty ml of peanut seed broth, sesame seed broth with maltose was prepared in 250ml glass conical flask. 5% of fresh pigment inoculums was added to each of the broth and incubated at 25^0C, 30^0C and 35^0C to study the effect of temperature on pigment production. The pH of the medium at the period to inoculation was uniformly adjusted to pH 7. Following 72 hours of incubation 1ml from each culture broth was taken for pigment extraction. Pigment production was estimated using spectrophotometer at 540 nm.

Effect of carbon sources on growth and production of prodigiosin by of *Serratia marcescens*

Nutrient broth with 0.5% maltose, nutrient broth with 0.5^0% glucose, 2% powdered peanut seed and sesame seed broth with 0.5% maltose in distilled water was prepared as 50ml in 250ml conical flasks the pH of the broth was maintained at 7. Maltose and glucose was filter sterilized and added to the respective media before inoculation. 5% of fresh pigment *Serratia marcescens* was added to each of the broth. The flasks were incubated at 25^0C, 30^0C and 35^0C to study the effect of sugar as a carbon source on growth of bacteria and pigment production. Following 72 h of incubation 1 ml from each culture media broth and the control medium was used for pigment production (Giri e*t al*., 2004).

RESULTS

Identification of isolates

The organism isolated from the soil was characterized as belonging to the genus *Serratia* by various biochemical tests, and other characteristics includes culture morphology, gram negative reaction, production of cell associated prodigiosin and lipase as well as non-lactose fermentation on Mac Conkey agar.

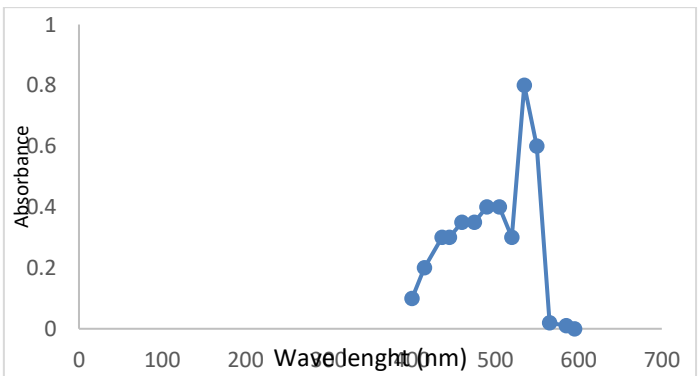

Figure 1. The UV/VIS Spectrophotometric scan of pigment produce

Table 1 Growth and production of prodigiosin by *Serratia marcescens* at 25ºC.

Media	Time (h)				
	24	48	72	96	120
Nutrient Broth with glycerol	1.30±0.12^{ab}	1.39±0.11^{b}	1.52±0.08^{a}	1.48±0.04^{a}	1.43±0.05^{ab}
Sesame seed broth with 0.5% maltose	6.32±0.55^{c}	7.22±0.14^{c}	8.43±0.48^{b}	7.40±022^{b}	7.30±0.07^{c}
Peanut seed with maltose	1.35±0.29^{ab}	1.46±0.05^{b}	1.62±0.10^{a}	1.52±0.04^{a}	1.51±0.02^{ab}
Nutrient broth with 0.5% glucose	1.31±0.06^{ab}	1.36±0.02^{b}	1.50±0.32^{a}	1.45±0.12^{a}	1.42±0.13^{a}
Peanut seed broth	18.31±0.40^{d}	20.40±0.48^{d}	25.10±1.61^{c}	23.10±1.30^{c}	22.30±0.66^{d}
Nutrient broth with 0.5% maltose	1.56±0.18^{b}	1.68±0.07^{b}	1.83±0.04^{a}	1.76±0.78^{a}	1.72±0.29^{b}
Peptone glycerol broth	0.51±0.04^{a}	0.62±0.08^{a}	0.85±0.06^{a}	0.78±0.04^{a}	0.71±0.06^{a}

Values with the same alphabets are not significantly different (p>0.05) along the column

The result above shows the significant level of various media used for the production of prodigiosin by *Serratia marcescens* at temperature of 25ºC. Peanut seed broth yielded the highest production at 72 h of incubation, which shows a significant difference with all other media.

Table 2 Optimization of pH for pigment production by *Serratiamarcescens*sp. UCP 1549

pH	Prodigiosin (ug/l)	Biomass (g/l)
2	0.00±0.00	0.00±0.00
3	0.00±0.00	0.00±0.00
4	0.00±0.00	0.00±0.00
5	16.00±1.27	1.8±0.06
6	15.00±1.80	2.00±0.11
7	25.00±1.47*	2.40±0.13*
8	15.00±2.10	1.60±0.06
9	16.00±2.05	1.80±0.10
10	0.00±0.00	0.00±0.00
11	0.00±0.00	0.00±0.00
12	0.00±0.00	0.00±0.00
13	0.00±0.00	0.00±0.00

Values are Mean± Standard Error of Mean of triplicate determinations. Value with asterisks (*) significantly higher (p<0.05) along the column.

Table 3 Optimization of incubation temperature for pigment production by *Serratia marcescens* spp. UCP 1549

Incubation temperature (^{0}C)	Prodigiosin (µg/l)	Biomass (g/l)
20	20.10±1.38	2.00±0.13
25	25.10±1.01*	2.50±0.17*
30	22.5±1.21	2.20±0.11
35	1.63±0.13	0.16±0.07
40	1.40±0.07	0.13±0.09

Values are Mean± Standard Error of Mean of triplicate determinations. Value with asterisks (*) significantly higher (p<0.05) along the column

Optimization of incubation temperature *Results presented in Fig.2 clearly show that pigment production by *Serratia* spp. UCP 1549 was maximum at 250C (25.10 µg/L) followed by 20^{0}C (18.56 µg/L). Whereas, pigment production decreased proportionately with increase in incubation temperature from 30^{0}C (15.20 µg/L) onwards .In general, it was observed that *Serratia* spp. UCP 1549 could produce the pigment at all the incubation temperatures tested and the values recorded for 15^{0}C was almost similar to that of 30^{0}C. Incubation temperatures above 30^{0}C led to a rapid decline in pigment production. Biomass production was maximum at 25^{0}C (2.5 g/L) and a gradual decrease was recorded after 72 h of incubation.

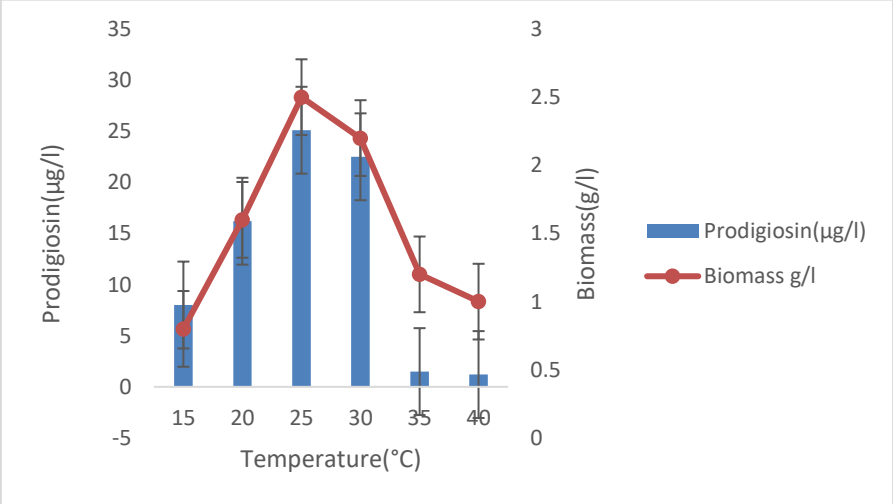

Fig: 2. Optimization of incubation temperature for pigment production by *Serratia* spp.UCP 1549: Fermentation conditions: 1 % inoculum, 150 rpm, 40 hof incubation and pH 7.0. Extraction was done in methanol at 50^{O}Cfor 20 min.

Optimization of initial pH of the fermentation media

Studies conducted for optimization of initial pH indicated that the bacterium could produce pigment over a range of pH from 5.0 to 9.0 although maximal pigment production was recorded at pH 7.0 (21.41 µg/L). It was also noted that pigment production declined along with increase in pH (Fig. 3). Media with high acidic (pH 2.0 - 4.0) and high alkaline (pH 10.0 - 13.0) reactions did not support pigment production. Biomass was maximum at pH 7.0 (2.4 gIL). Both pigment production and biomass showed a direct relationship, both in their increase as well as decrease, in response to variations in pH.

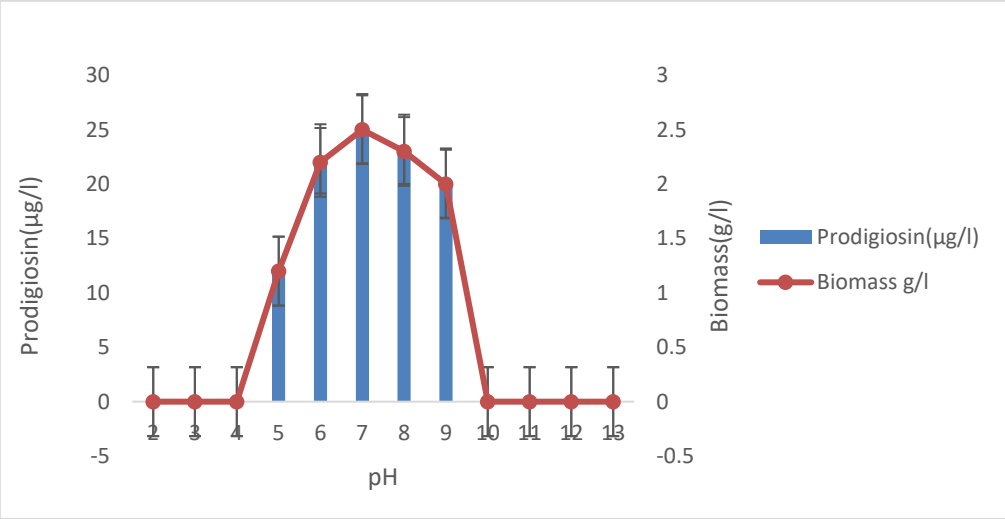

Fig: 3.Optimization of initial pH for pigment production by *Serratia* spp. UCP 1549: Fermentation conditions: 1% inoculum, 150 rpm, 48 h of incubation and incubation temperature 28±2^{0}C. Extraction was done in methanol at 50^{0}Cfor 20 min.

Effect of agitation

Agitation of the culture medium is required for pigment production by *Serratia* spp. UCP 1549 since there was no pigment production at static condition (Fig 4). The results showed that maximum pigment production was recorded at 150 rpm (22.50 µg/L) and the rate of agitation above and below 150 rpm led to a decrease in pigment production. Biomass also increased gradually during incubation from static condition to 150 rpm (2.5 g/L). This later decreased at 200 rpm. Hence, agitation at the rate of 150 rpm was required for maximal pigment production by this bacterium.

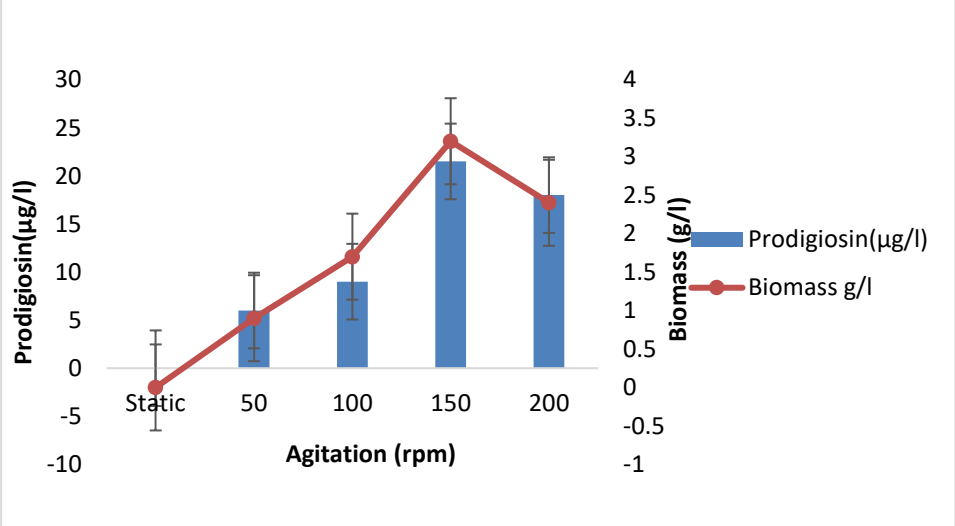

Fig: 4. Effect of agitation on pigment production by *Serratia* spp. UCP 1549: Fermentation conditions: 1 % inoculum, 48h of incubation, Incubation temperature 28±2^{0}C and pH 7.0. Extraction was done in methanol at 50^{0}C for 20 min.

DISCUSSION

Various environmental factors such as nutrients, temperature, pH and agitation influence the rate and amount of pigment production by bacteria. Hence, in order to increase the potentiality of the bacteria to synthesize maximal quantities of the pigment a suitable medium was developed using different media component known to be used commonly for carotenoid production. Among the different media evaluated, Peanut seed broth supported maximal pigment production (25.10 µg/l), when compared to Sesame seed broth, Nutrient broth, yeast extract peptone broth and Glycerol asparagine

broth. Since Peanut seed broth led to enhanced red pigment production by *Serratia* spp. UCP 1549, this medium was optimized further towards maximizing pigment production. The medium with optimum and minimum level of prodigiosin production is shown in Table 1. The present study agrees with the observation made elsewhere by Giri*et al*. (2004) that composed peanut seed broth and obtained an optimum production of prodigiosin (28.50µg/l). All proteins denature, or lose their optimal shape for carrying out a specific purpose, when heated. In some cases proteins that regulate the expression of other genes may not fully denature but lose their abil1ty to bind DNA when a certain temperature is exceeded. If the loss is reversible, these regulators regain the ability to bind DNA and activate or inhibit gene expression at lower temperatures. Another possibility is that temperature affects the activity of one or more enzymes involved in the synthesis of prodigiosin. At temperatures over 30^0C a particular enzyme in the pathway to synthesize prodigiosin may lose activity (http://www.madsci.org). In the present study maximum prodigiosin production by *Serratia* spp. UCP 1549 was observed at 25°C followed by 20°C and the production decreased proportionately along with increase in incubation temperature from 30°C to 40^0C (Fig.2) This is similar to observation made by Giri*et al.* (2004). Who recorded maximum yield of prodigiosin from *S. marcescens* at 28°C, while at 37^0C no pigment production was observed and the culture broth was white in colour. Maximal level of prodigiosin was synthesized by *S. marcescens*at 27^0C while no pigment was formed at 16 or 32^0C (Williams *et al.,* 1971). Further, it has been reported that the production of prodigiosin from *Serratia marcescens* was inhibited when the temperature was lower than 20^0C or higher than 37^0C (Furstner, 2003*;* Giri*et al.,* 2004*).* The present work is in contrast to observation made by Davis *et al.* (1957) *w*ho reported production of prodigiosin by some strains of *S. marcescens* when incubated at 37^0C

Extremes of pH, either acidic range (below pH 4.0) or alkaline range (above pH 10.0) prevented pigmentation by *S. marcescens* (Williams *et aI.,* 1971). While the optimum growth of all strains of *Serratia* has been observed at pH 9.0 (Giri*et al.,* 2004). The optimum pH for prodigiosin production by *Serratia marcescens* was observed at 8.0 (Wei *et al.,* 2005). In the present study, it was observed that the media pH influenced the pigment production by *Serratia*sp UCP 1549. Thus, there was considerable level of pigment production over a range of pH from 5.0 to 9.0 and maximal pigment was recorded at pH 7.0 in spite of a decline in pigment production along with increase in pH. Highly acidic (2.0-4.0) and alkaline (10.0 - 13.0) media did not support pigment production (Fig.3).

The agitation rate which influences the mass transfer of both oxygen gas and medium components in the medium become a crucial factor in prodigiosin synthesis by S. *marcescens*which recorded maximum pigment production at 200 rpm (Wei *et al.,* 2005). Prodigiosin biosynthesis by the nonproliferating cells was maximum when S. *marcescens* strain was incubated under aeration (Qadri and Williams, 1972). Mass transfer of medium components into the cells might play an important role in the relationship between agitation rate and prodigiosin production. Meanwhile, higher rate of agitation led to a slight decrease in pigment production, probably due to the problem of high shear force (Wei *et al.,* 2005). In the present study also it was observed that agitation significantly led to increase in the growth and pigment production by *Serratia* spp. UCP 1549. Maximum pigment production was recorded at 150 rpm and there was a decrease in pigment production at agitation rates above and below 150 rpm. Biomass also increased from static condition to 150 rpm.

CONCLUSION

Various environmental conditions influenced the growth of *Serratia marcescens*. Based on the different types of media used for prodigiosin yield, the saturated form of fatty acid as a carbon source could be a better choice of carbon as the maximum yield of pigment and biomass of approximately 25.10 µg/l and 2.5 g/l were observed in powdered peanut broth. Temperature of 25^0C was observed to be more suitable for the higher yield of prodigiosin as compared to other temperature studied. Neutral pH 7 gave maximum production of the pigment as compared to high acidic pH 2-4 and high alkaline pH 10-13 that did not support pigment production. Agitation enhanced prodigiosin production as higher yield was observed at 150 rpm as compared to other agitation levels. We have been successful in designing a economically feasible medium supporting the enhanced growth of *Serratia marcescens* and simultaneously supporting a high yield of medically important bio pigment, prodigiosin.

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