ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Probing natural carbon sources for bioactive pigment production from *S. nematodiphila* 213 C

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Manuscript Info

Manuscript History:

Received: 10 December 2013
Final Accepted: 28 January 2014
Published Online: February 2014

Key words:
Prodigiosin,*Serratia nematodiphila*, Biopigments

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Abstract

Prodigiosin is a hopeful drug outstanding to its reported distinctiveness of having antifungal, Immunosuppressive and anti-proliferative activity. Investigations made in the present study to construct prodigiosin by using different natural carbon sources as substrates such as cotton seed oil cake, ground nut seed oil cake, sesame seed oil cake, peptone glycerol broth, nutrient broth, sesame seed broth, nutrient broth with 1.5% maltose, peptone glycerol broth with 1.5% glucose, sesame seed broth with 1.5% maltose, sesame oil broth, peanut oil broth, soya oil broth were screened for prodigiosin production by using *Serratia nematodiphila* 213C. Sesame seed oil cake broth was more beneficial for prodigiosin production. In sesame seed oil cake broth maximum pigment production was obtained 1436.9 units/cell at 28^0C, pH 7.0, incubation time 72 h, 180 rpm with 2 % inoculums density. Exploit of sesame seed oil cake extract as a raw material for pigment production could be of immense marketable consequence. Further investigation of antimicrobial properties of prodigiosin revealed that it is a potent inhibitor against gram positive bacteria like *Bacillus cereus*, *Staphylococcus aureus*, gram negative bacteria like *E. coli*, and *Candida alb cans* like fungal pathogens.

Introduction

Pigments produced by organisms as reminiscence of its secondary metabolism are commonly referred as biopigments. These biopigments have wide synthetic and commercial application (Shirata A, 2000). A red pigment produced by many strains of the bacterium like *Serratia marcescens* and some other unrelated microbial strains, such as *Vibrio psychroerythrus*, *Streptomyces griseoviridis* and *Hahella chejuensis* was found to exhibit antibacterial, antimycotic, immunomodulating, anti-tumor and anti-malarial properties (Giri A, 2004). It also induces apoptosis in assured cancer cells (Furstner, 2003; Perez et al., 2003). The naturally occurring prodigiosin and prodigiosin like pigments subsist in cyclic and acyclic forms and contain 4 methoxy-2, 2' bipyrole ring (Manderville, 2001). Prodigiosin, itself an acyclic form, contains a 2 mehtyl-3-pentyl-pyrrole ring. The cyclic forms include metacycloprodigiosin, noncyclprodigiosin, streptorubin B and cycloprodigiosin (Manderville, 2001).

The utilization of natural pigments in foodstuff, dyestuff, cosmetic and pharmaceutical manufacturing processes has been increasing in recent years (Unagul P, 2005). Natural pigments can be obtained from two major sources, plants (Chang S; Nakamura, 1981) and microorganisms (Cho Y.J., 2002; Cross B. E., 1972). The accessible authorized natural pigments from plants have numerous drawbacks such as volatility against light, heat or adverse pH, low water solubility and are often non-availability throughout the year. The latter are of great interest owing to the stability of the pigments produced (Shirtata A, 2000) and the availability of cultivation technology (Kobayashi N, 1991). The advantages of pigment production from microorganisms include easy and fast growth in the cheap culture medium, independence from weather conditions and colors of different shades. Hence, microbial pigment production is now one of the promising fields of research to revel its potential for various industrial applications.

From an industrial point of view and because of its biological potentialities, there is a necessity to develop a high throughput and cost-effective bioprocess for large scale prodigiosin production. Conventional media used for the

biosynthesis of prodigiosin by *S. marcescens* strains are complex media that are rich in a variety of nutrients (Giri A., 2004; Harris K., 2004; Kobayashi N., 2004). Certain nutrients such as thiamine and ferric acid (Kim C, 1998b) are particularly crucial for prodigiosin production, whereas phosphate (Kim C., 1999), adenosine triphosphate and ribose (Iranshahi M, 2004) have inhibitory effects on prodigiosin yield. Giri et. al. (Harris K, 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. Earlier workers have reported many differential, selective and synthetic media such as, nutrient broth, peptone glycerol broth (PGB), LB broth etc, (Montaner *et al.,* 2000; Haddix and Werner, 2000) for prodigiosin and prodigiosin like pigment production. The yield of the pigment is varying: 3 g L-1 (Cang *et al.,* 2000) incorporating ethanol in the medium, 152 mg l-1 (Wei and Chen, 2005) in LB broth and oil supplements, and 0.08 g-4.28 gL-1 employing different carbon and nitrogen sources (Min-Jung-Song *et al.,* 2005). Giri *et al,* (2004) obtained 17 to 39 g L-1 yield and opned pea nut and sesame powder and their oils to be good substrates for prodigiosin biosynthesis.

Microorganisms differ in their needs to carbon sources according to their nutrient nature; the use of pure carbon sources e.g. (glucose, sucrose, and fructose) is expensive from cost-effective casing, so the industrial fermentation try to use contemptible carbon sources especially industrial and variety of plant seed oils have also been used as carbon substances for prodigiosin production and displayed stimulatory possessions on the production of pigment (Frustner A, 2003; Kim C, 2999). From an industrial point of view it is requisite to obtain a suitable medium to simultaneously improve the growth of organism and the pigment production.

According to the importance of prodigiosin in different applications, this study was aimed to produce prodigiosin from natural cost effective, ecofriendly carbon sources by local isolate of *Serratia nematodiphila* 213C to be able to assemble future needs.

Materials and Method

Microorganism

Serratia nematodiphila 213C was isolated from rhizospheric soil of sorghum (patil et.al., 2013), its logging number of Gene Bank was JN 166084, and was maintained on nutrient agar medium kept at 4^0C. Fresh cultures were obtained by inoculating nutrient broth medium with a loopful of stock culture, and incubated at 28^oC for 16 hours in an orbital shaking incubator 180 rpm.

Natural substrates for pigment production and culture medium

Nutrient broth, peptone glycerol broth, sesame seed broth, nutrient broth with 1.5 % maltose, peptone glycerol broth with 1.5 % glucose, sesame seed broth with 1.5 % maltose, sesame oil broth, soya oil broth, peanut oil broth, and the residue of cotton seed cake, sesame seed cake, and ground nut seed cake was collected from the local cotton seed oil industry, sesame seed oil industry, groundnut seed oil industry, North Maharashtra, Jalgaon. Except nutrient broth and peptone glycerol broth remaining natural substrates were obtained from local market for study of pigment production from *Serratia nematodiphila* 213C. The cotton seed cake, sesame seed cake and ground nut seed cake residue was dehydrated and crushed. To 100 ml water, 2gm of each cake powder was separately added and boiled for 20 min and allowed to cool. The supernatant was further centrifuged at 5000 rpm for 15 min to obtain a clear solution. The clear solution, after adjustment of pH 7.0 autoclaved at 121^0C for 15min. The remaining broth like nutrient broth, peptone glycerol broth, sesame seed broth, nutrient broth with 1.5 % maltose, sesame seed broth with 1.5 % maltose, peptone glycerol broth with 1.5 % glucose , sesame oil broth, peanut oil broth, soya oil broth etc. were prepared and sterilize separately and cool the all flasks. The flasks were inoculated with 0.5ml of 16h cell suspension of *Serratia nematodiphila* 213C and incubated at 28^oC in an orbital shaker at 180 rpm for 72h. After 72 h incubation estimate and extract the pigment yield. The media encompasses;

1. Nutrient broth (gL^{-1}) peptone, 10; sodium chloride, 5; yeast extract, 3
2. Peptone glycerol broth [meat extract 10 g/l, peptone 10 g/l, glycerol 10%]
3. 1.5 % powdered sesame seed in distilled water
4. Nutrient broth ($gL^{-1)}$ peptone,10; sodium chloride,5; east extract,3; with 1.5% maltose
5. Peptone glycerol broth with 1.5 % glucose
6. 1.5 % powdered sesame seed with 1.5% maltose in distilled water
7. 1.5 % sesame oil in distilled water

8. 1.5 % peanut oil in distilled water
9. 1.5 % soya oil in distilled water
10. 1.5 % Sesame oil seed cake broth in distilled water
11. 1.5 % ground nut oil seed cake broth in distilled water
12. 1.5 % Cotton oil seed cake broth in distilled water

Extraction of pigment

Pigment in the flask was extracellular, water diffusible though no need to extract pigment by solvent-solvent extraction. The flasks of fermentation broth were shaking at 180 rpm for 20 min at 28^0c. Debris was removed by centrifugation at 10,000 x g for 15min, and absorbance of approximately diluted samples was measured in UV – Visible spectrophotometer (Shimandzu., japan) at 535nm. The pigment content was calculated as unit/cell (Mekhael and Yousif, 2008)

Estimation of pigment

Isolated prodigiosin was estimated using the following equation (Suzuki T, 2001).

$$\text{Prodigiosin unit/cell} = \frac{[OD499 - (1.381 \times OD\ 620)] \times 1000}{OD\ 620}$$

OD 499 – pigment absorbance
OD620 – bacterial cell absorbance
1.381 – constant

Effect of pH on pigment production

Equal volume of the bacterial isolate was inoculated with various pH viz., 4,5,6,7 and 8.0. The flasks were incubated at 28°C for 72 h. The prodigiosin production was estimated after incubation. The initial pH at which maximum production of prodigiosin was observed was chosen and maintained in the following studies.

Effect of temperature on pigment production

Equal volume of the bacterial isolate was inoculated and incubated at different temperature viz., 24, 28, 32, 36 and 40 °C for 72 h. The prodigiosin production was estimated after incubation. The temperature at which maximum production of prodigiosin was observed was chosen and maintained in the following studies.

Effect of incubation on pigment production

To study the effect of incubation time on pigment production the isolate was cultivated in the presence of different incubation time viz., 18, 36, 54, 72, 90 h. After incubation the prodigiosin concentration was measured.

Effect of inoculums on pigment production

To study the effect of different inoculums concentration on pigment production the isolate was cultivated in the presence of different concentration viz., 1 %, 2%, 3%, 4%, 5%. The broth was adjusted to pH 7.0 fresh-pigmented culture was added to each of the broth. The flasks were then incubated at 28°C for 72 hours. After incubation the amount of prodigiosin produced in each medium was estimated.

Effect of Agitation

Bacterial isolate was inoculated into screened medium and added with different concentration of inoculums viz., 50, 100, 150, 180, 250 rpm. The prodigiosin unit/cell was estimated at each rpm.

Evaluation of in vitro antimicrobial activity of Serratia nematodiphila 213C water diffusible prodigiosin

Antimicrobial susceptibility assay

In vitro antimicrobial susceptibility assay was done following (Kobayashi, N; Ichikawa, Y., 1991) agar well diffusion method. The ATCC strains like Bacillus cereus, Salmonella typhi, Staphylococcus aureus, E.coli and Pseudomonas auriginosa, Candida albicans maintained in the Department of Microbiolgy, North Maharastra University, Jalgaon, Maharastra.

Sterile filter paper disc [6mm] were individually impregnated with 50ul of methanolic extract of prodigiosin. 95% methanol was taken as control. The entire disc were dried and placed on the surface of test bacterial and fungal lawn following 18 to 24 hours incubation at 37^0C the plates were examined for the zone of inhibition.

Results and Discussion

Serratia nematodiphila 213C was isolated from rhizospheric soil sample. In 1986, Grimont and Grimont proposed ribotyping as a general method for molecular bacterial identification. This method can also discriminate between isolates of the same species and it has proven to be a useful epidemiological tool in the study of various bacteria. 16S r DNA was amplified, sequenced and identified as Serratia nematodiphila. The 16Sr DNA sequence was submitted in GENBANK and the accession number is JN 166084. When the organism was allowed to grow in various media, the organism was found to produce more prodigiosin in sesame seed oil cake residue (Fig1). The prosperity of the pigment and its production was observed to be greatest in sesame seed oil cake broth. Differences in the color of the pigment produced in dissimilar substrates could be due to the compositions of the extracts of different substrates. Therefore, sesame seed oil cake broth was the favored choice-medium to bring out efficient investigations on medium enhancement and procedure optimization for the elevated capitulate of prodigiosin by Serratia nematodiphila 213C. The sesame seed oil cake broth which gave higher production of prodigiosin was further screened for effect of substrate concentration 1 to 5 %, pH, temperature, incubation and agitation.

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, pH and temperature ((Weinberg, 1970; Williamson et al., 2005). type of carbon source may play a decisive character in the Prodigiosin production (Furstner, 2003; Giri et al., 2004). Having an imminent on the composition of already published media, the idea of manipulating a new, nutritious and inexpensively cheap medium was attention of for the Prodigiosin biosynthesis and as significance, initial relative work was done using different carbon sources which included different types of sugars (glucose, maltose), 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 elevated 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 digression 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).

Nakamura [1986] has used triolein and reported a moderate yield of prodigiosin. In our study, the organism was found to produce 1436.9 units/cell of prodigiosin in sesame seed oil cake residues medium. This is comparatively lower than Nakamura [1986], but it is greater than Sundaramoorthy et al [2009]. Chang et al., [2000] has reported 3 mg/ml of prodigiosin when dextrose was used in the medium. Oller [2005] reported that glucose and sorbitol had a repressive effect on prodigiosin synthesis. Williams and Quadri [1980] reported that no prodigiosin was produced when cultures were incubated at 38°C; however pigment production was observed when the temperature was shifted to 25°C. A complete block in prodigiosin was observed in most of the basically used media tested at 37° C [Pryce and Terry, 2000]. Serratia nematodiphila 213C produces dark red coloured water diffusible, extracellular prodigiosin though no need to extract pigment by using different solvent. Therefore extraction of pigment from Sr. nematodiphila proves its econo value.

In natural environment Sr. nematodiphila 213C could produce red colored water diffusible pigment on the medium containing different carbon source. In our study, the residues from different oil seed industries was proven as an ideal replaceable for pigment production which contains abound nutrition. With some modifications, the prodigiosin yield of our study could be up to 1436.9 units/cell in sesame oil seed cake residual waste.

Table 1 Bioactivity of Prodigiosin

Staphylococcus aureus	1.2
B. cereus	0.13
E. coil	0.98
P. aeruginosa	-
Candida albicans	0.12
N. crassa	1.08

Fig 1 Production of prodigiosin by Serratia nematodiphila 213C in various media

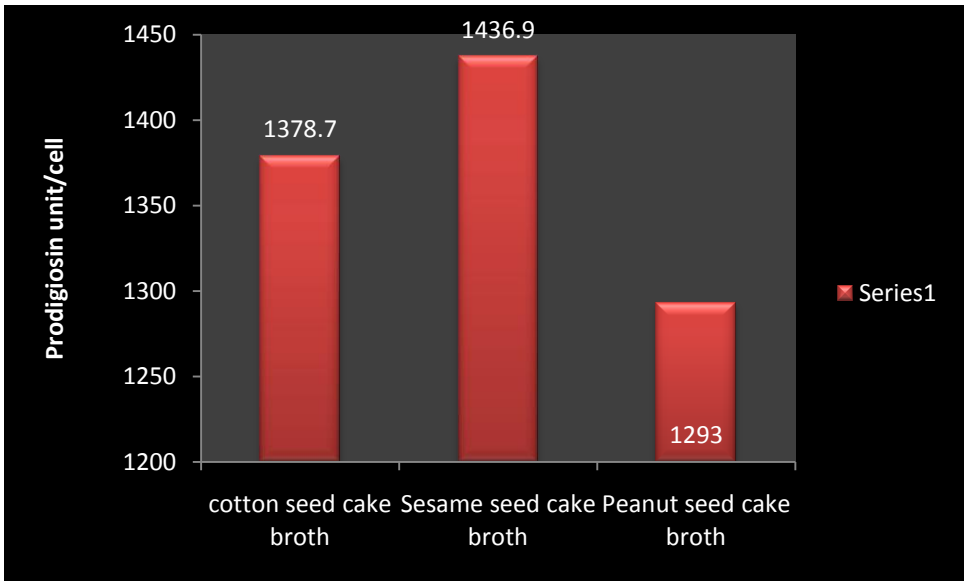


Fig 2 Production of prodigiosin by Serratia nematodiphila 213C in various media

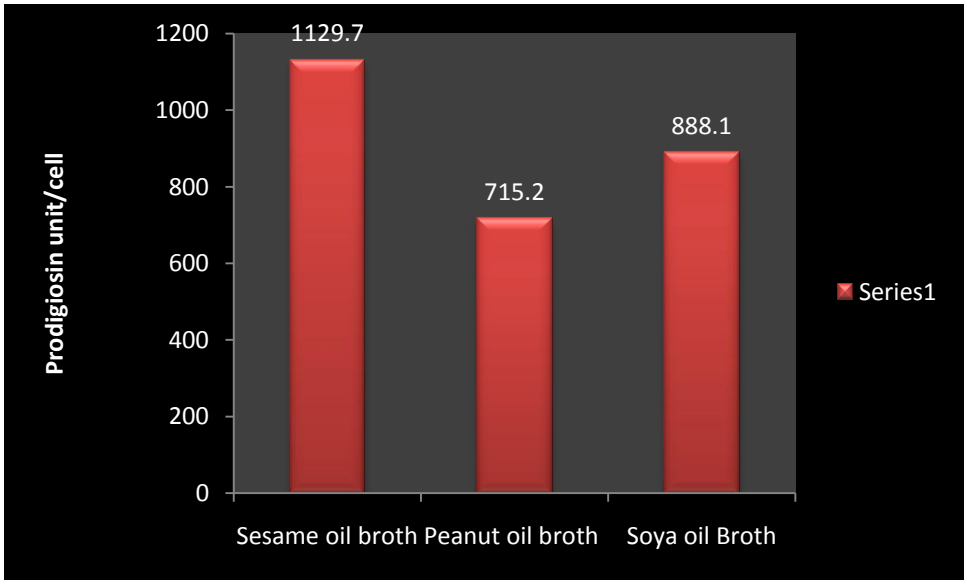


Fig 3 Production of prodigiosin by Serratia nematodiphila 213C in various media

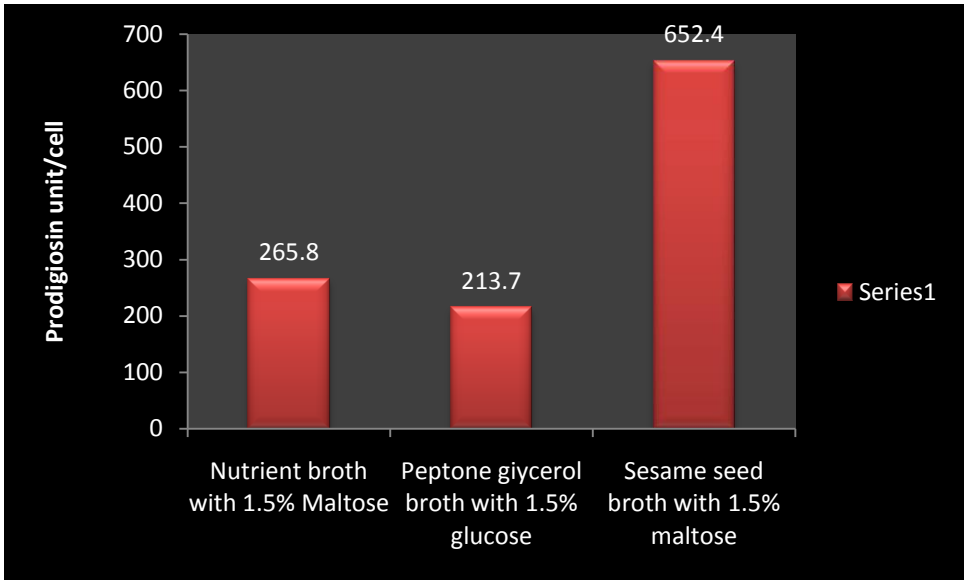
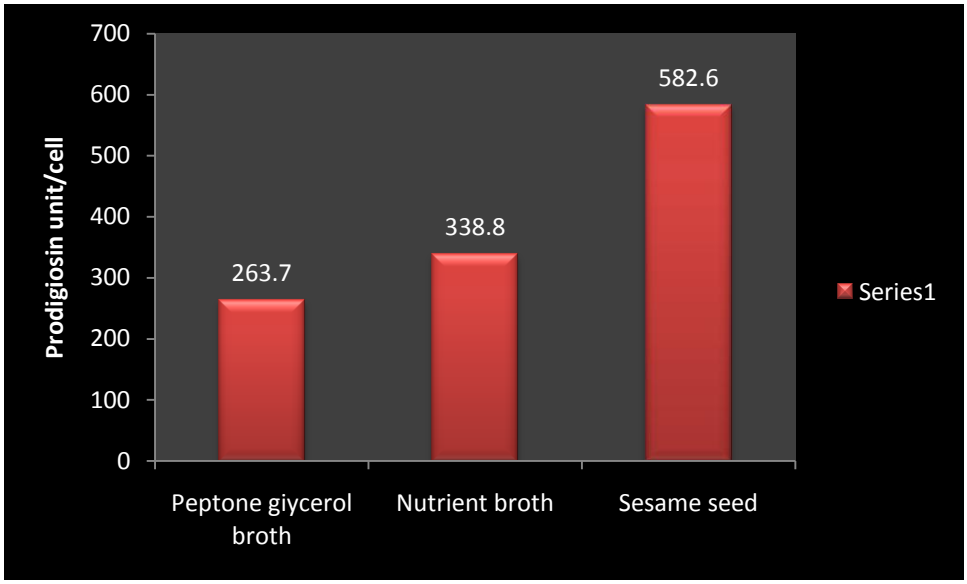


Fig 4 Production of prodigiosin by Serratia nematodiphila 213C in various media



Effect of pH

The influence of initial pH on prodigiosin production was investigated. Maximum yield of the pigment was obtained at pH 7.0 in sesame seed oil cake medium. Sole *et al.*, 1997 reported that the secondary metabolites are generally produced in the pH range of 6-8. Differences in the yield would depend on medium composition. Naik, et. al., 2012, reported that peanut seed oil cake extract naturally inherits oil as its carbon source and basically contains saturated and unsaturated fatty acids, carbohydrates, minerals, vitamins etc.

On the other hand, sesame seed oil cake broth naturally inherits oil as its carbon source and basically contains saturated and unsaturated fatty acids, carbohydrates, minerals, vitamins etc. **Effect of temperature**

Bacterial isolate was cultivated at different temperature and the amount of prodigiosin production maximum at 28°C in the sesame seed oil cake broth. The consequences of the influence of temperature on growth and pigment production by *S. nematodiphila* 213C were observed. Pigment production was totally blocked at 40°C in nutrient

broth (on as per with the report of Giri et al., 2004) and after 72^{o}C in the sesame seed oil cake broth. The impact of physiological role of temperature in blocking prodigiosin synthesis seems to diverge with mediums of different substrate composition (Giri et al., 2004).

Effect of incubation period

Incubation period plays a very imperative role in the production of bacterial secondary metabolites. Incubation period varied in the range of 18 to 72 h. Pigment production in progress from 18th h of growth in sesame seed oil cake broth and it was linear from 18th to 72nd h in case of sesame seed oil cake broth. The sesame seed oil cake broth is a complex and undefined nutrient source of saturated and unsaturated fatty acids yielding carbon, proteins, minerals, vitamins and hence, the delay in pigment production arrival might have been caused.

Effect of inoculum density

Lower initial inocula in the broths led to lower prodigiosin production when compared with higher initial inocula. Pigment production was maximum with 2 % initial inoculum density in sesame seed oil cake broth. Maximum pigment production was observed at 2 % (W/V) of sesame seed oil cake extract. But with higher substrate concentrations, biomass production alone was benefited, not pigment production. Probably higher substrate concentrations suppress prodigiosin production. The prodigiosin production was maximum in 72 h. Pigment expression mainly depends upon quorum sensing phenomenon (Fuqua *et al.,* 1996).

Study of different natural sources of substrates for production of pigment by *Serratia nematodiphila* 213C.

Following incubation of the different natural sources of substrates for pigment production at 28^{0}c, 180 rpm for 72 hours, the samples analysed for extracellular pigment production. The medium containing residues of sesame seed oil cake gave maximum yield of prodigiosin at 28^{0}c amongest the three oil seed cake residues medium. When compared to sesame seed oil cake medium cotton seed oil cake medium and ground nut oil seed residues gave a half fold decrease in pigment production at 28^{0}C, 180 rpm and 7.0 pH. The descending order of pigment production was sesame seed oil cake medium, ground nut seed oil cake medium, cotton seed oil cake medium, sesame seed oil broth, peanut nut seed oil broth, soya seed oil broth, nutrient broth with 1.5 % maltose, peptone glycerol broth with 1.5 % glucose, sesame seed broth with 1.5 % maltose, peptone glycerol broth, nutrient broth, sesame seed broth. The maximum yield of pigment in case of sesame seed oil cake medium is 1436.9 units/cell.

In vitro bioactivity potency

The extraction was found to have antibacterial activity by the use of disc-agar diffusion technique, it was observed that the prodigiosin was able to inhibit majority of the test bacteria. As indicated in [Table 1]. The inhibitory zones for bacteria varied between 0.13 mm and 1.2 mm, whereas, fungicidal activity was evident from the clear zones of inhibition observed against *C. albicans* and *N crassa*. Prodigiosin possesses antibacterial activity against gram positive bacteria like *Staphylococcus aureus*, gram negative bacteria like *E.coli* and *Pseudomonas* species. The fungal pathogens being sensitive to this potent antimicrobial agent *C.* albicans, N. crassa to a lesser extent *C. albicans* (Khanafari *et al*., 2006). Moreover, soild fermentation methods could save the cost of maintaining and operating normal fermentation equipments and shorten the refining process.

Conclusion

In order to increase the potentiality of the bacteria to synthesize large quantities of the pigment a comparative study of different media, growth of the organism in the different media and pigment production must be studied. The final conclusion based on the experimental results could be that the saturated fatty acid form of carbon source is a better substrate for the growth of *Serratia nematodiphila 213C* than sugars and unsaturated fatty acids. Based on the comparison between the composition of the different fatty acid containing seeds, oils and oils seed cakes in terms of 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 of approximately 1436.9 units/cell was seen in the case of sesame seed oil cake broth. We have been successful in designing an economically feasible medium supporting the enhanced growth of *Serratia nematodiphila* 213C and simultaneously supporting a high yield of medicinally important biopigments, prodigiosin. The significance of the present study is that an economically cheap and easily available by products can be used, without any external addition of nutrients for prodigiosin production using sesame seed oil cake as substrate for fermentation. Studies are in progress for large scale pigment production employing *Serratia nematodiphila* 213C using sesame seed oil cake broth as the substrate.

Acknowledgment

Author is thankful to North Maharashtra University, Jalgaon for G. H. Raisoni Research fellowship.

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