International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Special Issue-2 (2015) pp. 254-267**
http://www.ijcmas.com

Original Research Article

Comparative Studies on Prodigiosin Production by *Serratia marcescens* using Various Crude Fatty Acid Sources-Its Characterization and Applications

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A B S T R A C T

Pigment producing organisms were isolated from various sources and screened for their ability to produce pigment. Isolate PP1 was selected for further studies because of higher pigment yeilds. It was biochemically characterized and identified to be *Serratia marcescens* by API 32 GN kit. Production of prodigiosin was optimized with respect to different environmental parameters such as pH and temperature. Various crude and pure carbon sources in form of peanut, sesame, castor, coconut and sunflower seeds were used for enhancing production. The powdered peanut broth and sunflower seed broth supported better growth of *Serratia marcescens* and showed higher yield of prodigiosin. Effect of different amino acids such as proline, cystein, methionine and combination of all three on prodigiosin production was also studied along with effect of addition of glucose and maltose. A combination of amino acids at different concentrations gave highest yield at pH 6. Acetone gave highest yield for extraction of prodigiosin. Crude acetone extract of pigment was further purified by subjecting it to preparative thin layer chromatography. Prodigiosin was tested for range of applications such as antibacterial and antifungal activity, plasmid curing potential, biosurfactant and bioemulsification activity, anticancer potential and immunosuppressive activity. The purified pigment inhibited both Gram positive and Gram negative pathogens used for testing as well as growth of *Serratia* on chitin agar showed antagonism against test fungi. Pigment also showed positive results for plasmid curing and bioemulsification activity. Prodigiosin in 20% DMSO also showed anticancer activity against MCF7 cell lines using MTT assay but failed to show any immunosuppression activity.

Keywords

Prodigiosin, *Serratia*, Crude fatty acids

Introduction

Prodigiosin is a red colored pigment belonging to the family prodiginines and has a tripyrrole in its structure (Venil *et al.*, 2009, Grimont and Grimont, 1978).

It is reported to be produced by species of *Serratia, S. marscesens, S. plynuthica* and *S. rubidaea* and actinomycetes belonging to genus *Streptomyces*. Apart from these, a range of other bacteria including

Pseudomonas magnesiorubra, Vibrio psychroerythrus and *γ-Proteobacteria* also produce this bright pigment (Pandey *et al.,* 2009, Bennett and Bentley 2000).

Prodigiosin is of special interest in research because this molecule shows a variety of important activities such as antifungal, antibacterial, plasmid curing, anticancer, immunosuppressive, anti tumor, anti neoplastic and antioxidant activity. Prodigiosin produced by *Serratia marcescens* is reported to have antifungal, immunosuppressive and antiproliferative activity (Giri *et al.,* 2004). The antifungal activity of the crude prodigiosin, separated through solvent extraction process and purified by TLC was tested against fungal pathogens following the well-diffusion method, which showed the maximum inhibitory zone against *Helminthosporium sativum, Fusarium oxysporium* and *Rhizoctonia solani* in decreasing order (Parani and Saha 2008). There are many factors involved in the anticancer and immnosuppressive activity of prodigiosin (Kavitha *et al.,* 2010, Pandey *et al.,* 2007). Pandey *et al.,* have given an extensive account of literature on the same (Pandey *et al.,* 2009). Pyrrol ring has been reported to be very important in activity and prodigiosin structure analogues have also been designed for therapeutic use (Frustener 2003, Manderville, 2001).

In an interesting study by Murugkar *et al.* (2006) a red coloured microbe was isolated from mangrove soil. Large amounts of red color were produced on Modified Nutrient agar. This dye was used for dyeing natural fibres such as silk, wool and synthetic fibers like polyester and gave a good pink color. Standard procedure of dyeing was followed for silk, wool and polyester. Good colorfastness of the dyed substrates to washing, sublimation and rubbing was observed but the color easily faded when dyed material was exposed to light. However since the dye displayed an antimicrobial activity against *Staphylococcus aureus, Corynebacterium diphtheriae, Nocardia spps* and *Micrococcus luteus,* its commercial value increases (Murugkar *et al.,* 2006). Purified prodigiosin has also been reported to show plasmid curing activity on plasmids of *E. coli* HB101 and *S. aureus* (Mekhael and Yousif. 2009).

All these important applications signify study on better production and purification of prodigiosin. A medium containing powdered peanuts has showed forty fold increase in the concentration of prodigiosin (Giri *et al.,* 2004). A comparative account of effect of different nutrient sources on prodigiosin production is published by Khanafari et al. (2006) where they have also elaborated on genetics of prodigiosin production, block in synthesis, various activities and toxicity of this pigment (Khanafari et al., 2006). Pigmented *Serratia* have been shown to cause infections in much less frequency than non pigmented *Serratia*, thus reducing the risk of infection during mass production of pigment (Carbonell *et al.,* 2000)

In present study we have tried to increase the production of prodigiosin from *Serratia marcescens* by using different media as well as purified prodigiosin is tested for various activities.

Materials and Methods

Isolation of pigment producing bacteria from different sources

Beach sand samples from coastal area of Kashid and Alibaug, rhizosphere soil sample of Modern College, Pune 5, botanical garden, air sampling, pond water sample from Modern College, Pune 5 and fish gut

samples were used for isolation. One gram of soil sample collected from different sites was mixed in 10 ml of sterile saline. Serial dilutions of sample were spread on sterile Nutrient agar plates. For air sampling, plates were directly exposed to air. After incubation at 28^0C for 24 hours plates were observed for pigmented colonies. Colonies showing reddish pink pigment were purified on Nutrient agar plates. Colony characteristics were studied for these isolates and those which were presumptively identified as *Serratia* using Bergey's manual of Determinative Bacteriology (9th edition) were taken for further study. Pure cultures were preserved on Nutrient agar plates at 4^0C till further use.

Preliminary identification of the pigment

Presumptive color test for Prodigiosin was carried out by scraping the pigmented growth on 10% Milk agar medium plates. 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 in acidified ethanol (10%); the other part was alkalinized in acetone solution (20%) (Ding *et al.,* 1983).

Screening of prodigiosin producing bacteria

General screening procedure was used for screening maximum pigment yielding i.e prodigiosin producing bacteria by inoculating a loop full of culture in Nutrient broth followed by 24hr incubation. Spectrophotometric quantitation of extracted pigment was followed for screening.

Characterization of isolate by API 32GN KIT

After screening, one isolate PP1, giving highest production of pigment, was selected for further study and was identified upto genus and species level by using API 32 GN kit.

Enzymatic characterization of isolate PP1

Isolate PP1 was extensively characterized for different enzymes such as gelatinase, urease, nitrate reductase, chitinase. Hemolysis was also studied on 5% (v/v) fresh human whole blood agar plates.

Inoculum development for large scale production

The preparation of a population of microorganisms from a dormant stock culture to an active state of growth that is suitable for inoculation in the final production stage is called inoculum development. As a first step in inoculum development, culture was taken from working stock to initiate growth in a suitable liquid medium. Inoculum development was generally done using flask cultures; flasks of 500 ml and 1litre were used. For all experiments described below for optimization of medium and conditions, inoculum was first optimized using 1–5% (v/v) of the production medium.

Medium optimization

Fatty acid sources

Crude fatty acid sources like Peanut, Groundnut, Sesame, Castor and Sunflower seeds were surface sterilized using $HgCl_2$ solution. Seeds were then crushed using surface sterilized motor and piston. 2 % of each seed powder added in distilled water separately.1% (v/v) of fresh inoculum was added in each broth. Uninoculated controls were kept for each experiment to assure sterility after autoclaving. All test and controls were incubated at 28^0C in the Incubator shaker. Optical density was taken

at 540 nm daily till there was decrease in optical density. Highest yield of prodigiosin was observed in peanut broth followed by Sunflower seed medium. Thus the environmental factors are optimized for peanut medium as well as further for sunflower seed medium. Since peanut broth is reported earlier, we selected sunflower seed medium for optimization of amino acids to check its effect on production.

Effect of physico-chemical factors on prodigiosin production

The effect of pH on prodigiosin production was studied. Peanut broth was adjusted to initial pH (6, 7, 7.5 and 8) and inoculated with *Serratia marcescens.* To observe the effect of temperature on prodigiosin production, peanut broth was inoculated with *Serratia marcescens* and incubated at different temperatures (28^0C, 32^0C, 37^0C and 42^0C). Effect of agitation on prodigiosin production was detected by incubating inoculated media at different agitation conditions (120, 140, 160, 180 rpm). After 48h pigment was extracted and purified to check the effect on prodigiosin yield in all above experiments.

Effect of sugars and amino acids on prodigiosin production

2% Sunflower seed powder added in 3 flasks containing 0.5% of Glucose and cystiene, methionine, proline was membrane filtered and each was added in 3 different concentrations. For each amino acid different concentration was selected. For cystiene 0.0015%, 0.0030%, 0.0045%; for methionine 0.0050%, 0.010%, 0.015%; for proline 0.05%, 0.15%, 0.25% was added separately in different flasks. These concentrations were selected based on previous reports on prodigiosin production. 1% v/v of inoculum was added in each flask.Incubated at 28^0 C in incubator shaker and absorbance at 540 nm was measured on 6th day.

Designing pigment purification protocol

Extraction

Serratia marcescens grown in powdered peanut broth and sunflower seed broth was centrifuged at 10,000 rpm for 15 minutes and the supernatant and cell pellet were extracted with ethyl acetate, petroleum ether, diethyl ether, acetone and methanol. Cell pellet was repeatedly subjected to extraction till a white pellet was obtained. The pigment extracts of ethyl acetate fraction and acetone fraction were evaporated separately in porcelain evaporating dishes at room temperature till a powdered form of pigment was obtained.

Purification

The crude pigment extracted from culture broth was further purified using preparative thin layer chromatography. Solvent system, used for effective separation of impurities was dichloromethane: chloroform: acetone (2.5:2.5:0.5). 10% crude pigment solution in methanol was spotted on a thin layer chromatography sheet. Spots of pigment purified by TLC were scraped and extracted in methanol. The extract was transferred into clean vials and centrifuged at 10,000 rpm for 10min to separate silica and pure pigment. The supernatant contained purified pigment and was transferred into porcelain evaporating dishes and was evaporated at room temperature till a powdered pigment was obtained. The pure pigment extracted from the culture broth was taken for spectrum analysis in range on 200 to 700nm using UV spectrophotometer to check the purity.

Applications of prodigiosin

Antibacterial activity

Antibacterial activity of prodigiosin pigment was studied against different species of Gram positive bacteria *Staphylococcus aureus, Bacillus subtilis* and Gram negative bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Proteus sp.* and *Klebsiella pneumoniae.* The test cultures were spread on Muller Hinton's agar plates and activity of Prodigiosin was studied using well diffusion method. Acidified ethanol was used as control.

Antifungal activity and fungal antagonism

Antifungal activity of prodigiosin was checked against different species of fungal pathogens. The fungi included were *Aspergillus niger, Mucor* sp and *Rhizopus* sp. The cultures were spread on Potato Dextrose Agar (PDA) plates and activity of prodigiosin was checked using bore well technique. The plates were incubated at 28ºC for 48h. Control used was acidified ethanol (10%). Antagonism between *Serratia* and fungi was determined by spot inoculation of fungal pathogen on one side of 2% potato dextrose agar plates and chitin agar plates containing swollen chitin (0.4%) and incubated at 28^0C for two days. After two days of incubation/growth, a loop full of overnight culture of *Serratia marcescens* (PP1) was streaked on opposite side of fungus grown potato dextrose agar plates and chitin agar plates. The diameter (mm) of the zone of inhibition between the bacteria and fungus was used as an indication of the extent of antagonism.

Plasmid curing

The effect of prodigiosin pigment as a curing agent was tested to cure plasmid of different bacteria as *E. coli*, *P. stuartii* and *S. aureus.*100ml of LB broth was inoculated with the test organism which showed plasmid encoded resistance to specific antibiotic (Gentamycin in case of *P. stuartii*) and incubated for 24h. Different concentrations of prodigiosin pigment were prepared ranging from 0.2 mg/ml to 1 mg/ml. 1ml of culture suspension of each test organism was added to 1ml of each dilution of prodigiosin. Tubes were incubated for 1h at room temperature. After 1h of incubation 0.1ml suspension from each dilution tube was spread on sterile Luria agar plates containing antibiotics to which particular organism was found to be resistant. Plates were incubated at 37^0C for 24h. After 24h plates were observed for the presence/absence of colonies.

Bioemulsification activity

Serratia marcescens (PP1) was studied for its emulsification activity. 100ml of LB broth was inoculated with *Serratia marcescens* (PP1) and incubated for 24h. After 24h incubation, the broth was centrifuged and this cell free broth was further used for checking emulsifier activity. Emulsifier activity was measured by adding 6 ml of kerosene to 4 ml of cell free broth and vortexing at high speed for 2 min. Measurements were made 24h later. The emulsion index (E_{24}) is the height of the emulsion layer, divided by the total height, multiplied by 100.

$$\text{Emulsion Index } (E_{24}) = \frac{\text{Height of emulsion layer}}{\text{Total height of broth}} \times 100$$

Anticancer activity (Cytotoxicity studies) of prodigiosin by MTT assay and Immunosuppressive activity

MCF7 (human breast adenocarcinoma) was cultured in a humidified atmosphere (37°C,

5% CO_2) in RPMI1640 medium supplemented with 10% fetal bovine serum.

Test compounds were evaluated for antiproliferative activity against MCF7 cell lines using the MTT colorimetric assay was used to determine growth inhibition. 100 µl of cell suspension ($2x10^4$ cells/well) were plated in 96-well plates and allowed to attach for 24 h. Cells were exposed in triplicate wells to test compounds at various concentrations for 24 h. 20 µL MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) solution (5 mg/mL,) was added to each well.

After 4 h of incubation, the formazan was dissolved using solubilization buffer (10 % SDS in 0.1 N HCL) solution and kept overnight for complete dissolution. The absorbance at 570 nm was determined on a microplate reader. The absorbance values were used to calculate % inhibition (114,115). Prodigiosin was also studied for immunosuppressive activity on lymphocyte cell lines.

Results and Discussion

Isolation and characterization of prodigiosin producing bacteria from different sources

About eight pigment producing bacteria were isolated from soil samples and air sampling. Preliminary screening was carried out to identify a profusely pigmented (prodigiosin- a red pigment producing) bacteria. The screened isolate i.e. PP1 (Figure 1) was selected for further work.

Preliminary identification of the pigment

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 (Figure 2).

Characterization of isolate by API 32GN KIT

Bacterial isolate (PP1) selected for further studies was biochemically characterized and identified to be *Serratia marcescens* by API 32GN kit (Figure 3).

Biochemical characterization of *Serratia marcescens* PP1

PP1 isolate was found to ferment various sugars (table 2) and produce different enzymes (table 3)

Isolate used for further study was checked for gelatinase, urease, nitrate reductase and chitinase enzyme production and hemolytic activity. The isolate showed positive result for all the enzymes checked and also showed β-hemolysis on blood agar plate.

Inoculum development for large scale production

Maximum yield of the pigment was obtained at 1-3% inoculums which decreased for next higher concentrations.

Medium optimization:

The bacterial isolate was cultivated in medium amended with powdered peanut, sesame, coconut and castor seed. Maximum yield was obtained in medium supplemented with peanut seed i.e. powdered peanut seed broth as a fatty acid source. The results obtained are represented graphically which shows that maximum yield was obtained in powdered peanut broth which gave the highest yield i.e. 1595.09mg/L after 48h (Figure 5).

The pH affects the production of prodigiosin by *Serratia marcescens* (PP1). This species showed maximum prodigiosin production at pH 7 (1398.06mg/L). There was less

prodigiosin production at pH 6 and 8. *Serratia marcescens* showed maximum prodigiosin production at 28^0C. Following incubation of powdered peanut seed broth at different temperatures (28°C, 32°C, 37°C and 42°C) at 120 rpm for 48h, the prodigiosin yield obtained is represented in graph. As indicated from graph the medium containing powdered peanut seed gave maximum yield of prodigiosin at 28°C, 32°C, and 37°C.

Amongst the three temperatures maximum yield was observed at 28°C in powdered peanut seed medium (Figure 6). Sunflower seed media with maltose showed 1.556 mg/ml prodigiosin production while when supplemented with glucose showed 1.525 mg/ml prodigiosin production on 6th day which was highest.

Since sunflower seed medium showed maximum production of prodigiosin in presence of maltose as compared to other media, it was selected for further optimization using amino acids. Sunflower medium in presence of maltose and with 1.5mg of cysteine, 15mg of methionine, 250mg of proline showed maximum prodigiosin production. Highest pigment production at 28°C and pH 6.0.

Extraction of prodigiosin

The supernatant was extracted in ethyl acetate, petroleum ether, diethyl ether and methanol. None of the extracts gave residual crude pigment.

The pellet was extracted in acetone which yielded a residual crude pigment. From 800ml of nutrient broth containing prodigiosin, the pellet residues gave a yield of 0.31g. Thus acetone was found to be best for extraction of pigment from pellet. The amount of partially purified pigment was found to be 0.52mg/100ml.

Applications of prodigiosin

Antibacterial activity

Results shown in table 4 and figure 10 indicated that the prodigiosin antibacterial activity was higher against Gram positive bacteria including *Staphylococcus aureus and Bacillus subtilis* as compared with Gram negative bacteria such as *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae.*

Antifungal activity

In Chitin supplemented agar medium, the growth and production of red pigment by *Serratia marcescens* (PP1) was highly antagonist to the fungal pathogens like *Alternaria alternata, Aspergillus niger* and *Fusarium oxysporum*. But when pure prodigiosin was tested for antifungal activity, it revealed that the fungal species, *Aspergillus niger, Mucor sp* and *Rhizopus sp* were resistant to the pigment and showed no zone of inhibition.

Plasmid curing

P. stuartii was found to be sensitive to gentamycin after plasmid curing. Prodigiosin showed curing of plasmid at all concentrations used i.e. from 0.2 mg/ml to 1 mg/ml.

Anticancer activity (Cytotoxicity studies) of prodigiosin by MTT assay and Immunosuppressive activity

The test compound showed an enhanced effect of 56.03 ± 3.47 % as compared to its half concentration tested which showed 33.62 ± 3.47 % inhibition against MCF 7 cell lines after 24 hrs of incubation. Thus it can be concluded that prodigiosin has anticancer activity. Compound showed no

effect on lymphocyte cell lines and thus is not immunosuppressive.

Table.1 Morphological characteristics of isolate PP1

Morphological characters	Observation
Size	0.2-0.5µm
Shape	Circular
Colour	Red
Margin	Entire
Elevation	Convex
Opacity	Opaque
Consistency	Smooth
Motility	Motile
Gram character	Gram negative

Table.2 Biochemical characterization of PP1

Sugars	Result	Colour change	Gas production
Lactose	+	Red to Yellow	-
Maltose	+	Red to Yellow	+
Glucose	+	Red to Yellow	-
Fructose	+	Red to Yellow	+
Sucrose	+	Red to Yellow	-
Mannitol	+	Red to Yellow	-

Table.3 Enzyme profile of Serratia marcesens PP1

Enzymes	Results
Gelatinase	+
Urease,	+
Nitrate reductase	+
Chitinase	+

Note: + = Positive

Table.4 Antibacterial activity of prodigiosin against Gram positive and Gram negative bacteria

Test organism	Diameter of Zone of inhibition(mm)
Staphylococcus aureus	16
Bacillus subtilis	15
Escherichia coli	10
Klebsiella pneumoniae	12
Pseudomonas aeruginosa	8
Proteus sp	12
Salmonella typhi	No zone

Table.8 Bioemulsification activity of prodigiosin with different Oils/ Hydrocarbon

Oil/ Hydrocarbon	E_{24} Test
Kerosene	59.25
Toluene	58.33
Xylene	62.5
Coconut oil	8.69
Sunflower seed oil	5.70

Figure.1 Isolate PP1

Figure.2 Preliminary identification of Prodigiosin (A: Ethanol treatment, B: Acetone treatment)

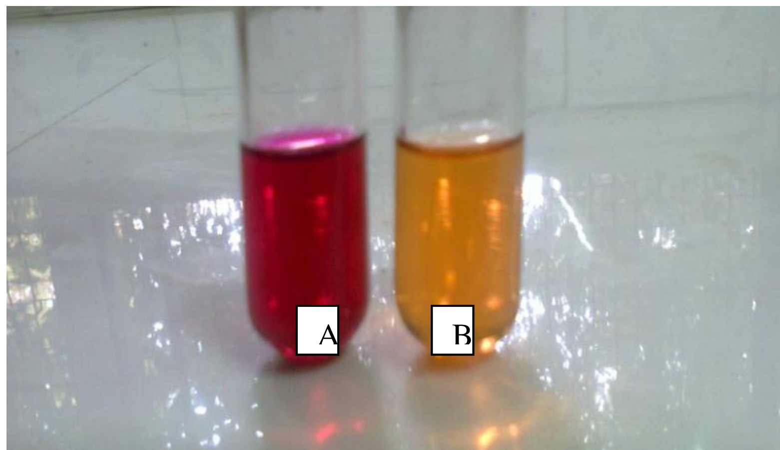

Figure.3 Characterization and identification of isolate PP1 (*Serratia marcescens*) by API 32 GN kit

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PATIENT        DISPLAY        COMM.        EXPERT        UTIL.        api/MATT
Patient No.ID
PATIENT        DISPLAY        COMM.        EXPERT        UTIL.        api/MATB
Patient No.ID11
 Date of receip|      Specimen No.ID110/10                    2* organism
               |
Surname:X      | Organism:Ser.marcescens
               | Identification strip:ID 32 GN              V3.1
Number of specin| API Profile:
               |RHA  -  NAG  +  RIB  +     IND  +  SAC  +  MAL  +
               |ITA  -  SUB  -  MNT  -     ACE  ?  LAT  +  ALA  +
  Reference    |MAN  +  GLU  +  SAL  +     MEL  -  FUC  +  SOR  +
               |ARA  -  PROP -  CAP  +     VALT -  CIT  +  HIS  +
               |5KG  +  GLYG =  mOBE =     2KG  +  3OBU =  pOBE =
ID110/10       |SER  +  PRO  +
Reference: ID118/10
 X x
Requested by:
Specimen
SOURCE:
Profile: -+++++---?+++++-++--+-++++---+----++
ID 32 GN          V3.1 : VERY GOOD IDENTIFICATION OF THE GENUS
Ser.marcescens            %id= 86.1   T= 0.80

Ser.liquef./plymuth.      %id= 13.9   T= 0.50
MEL   98%  ARA   98%
NEXT CHOICE
Ent.cloacae               %id=  0.1   T= 0.07
RHA   88%  MEL   85%  ARA   98%  CAP   9%  5KG   2%
POSSIBILITY OF Serratia ficaria

>>>>Selected taxon: 1 Ser.marcescens O
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Figure.4 Effect of Inoculum size prodigiosin production

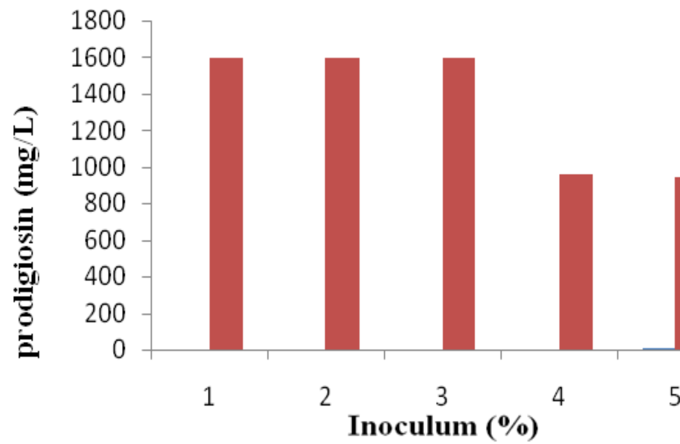


Figure.5 Effect of crude fatty acid sources on prodigiosin production

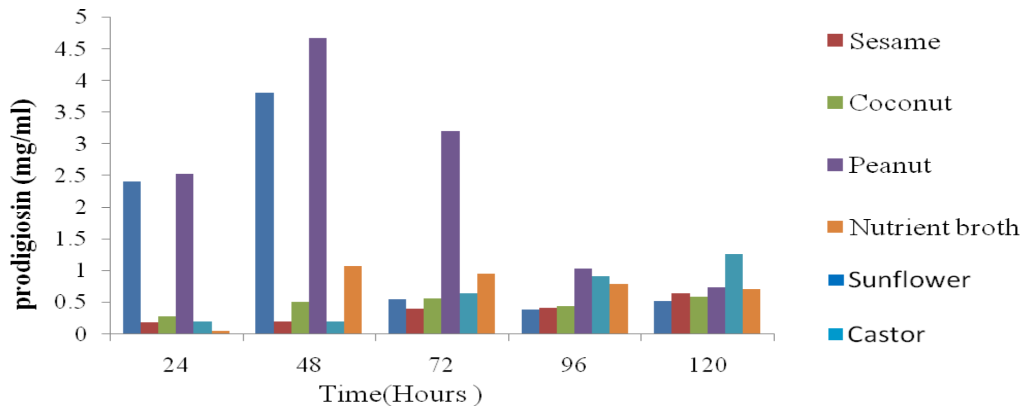


Figure.6 Effect of physico-chemical factors on prodigiosin production

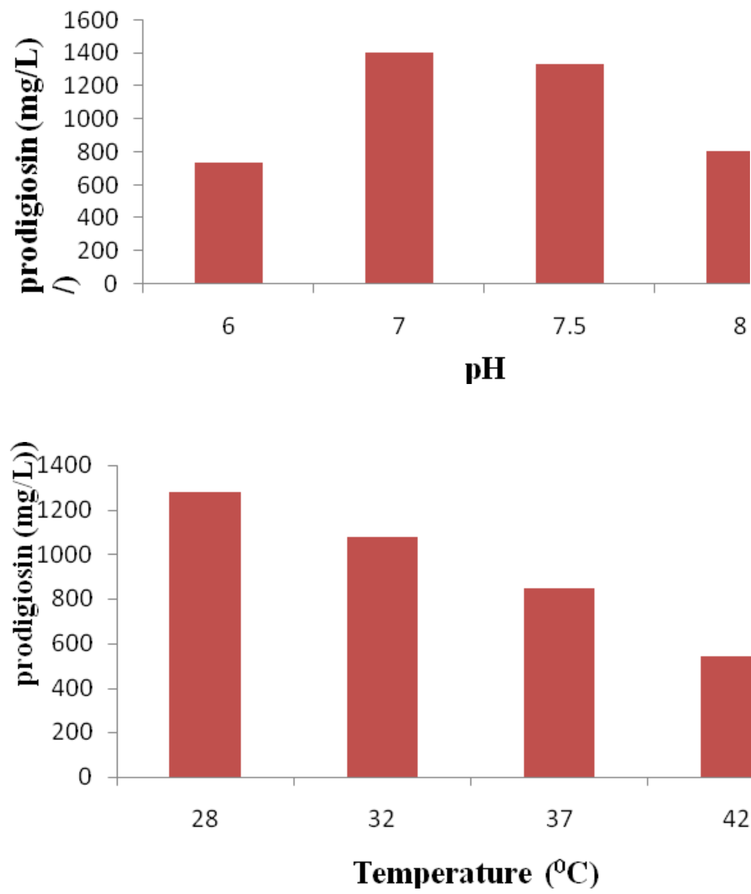


Figure.7 Effect of Glucose on prodigiosin production with different crude fatty acids sources

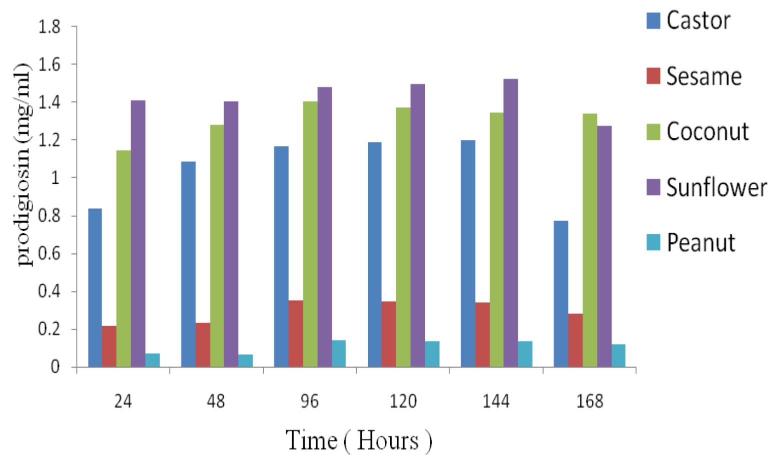


Figure.8 Effect of Maltose on prodigiosin production with different crude fatty acids sources

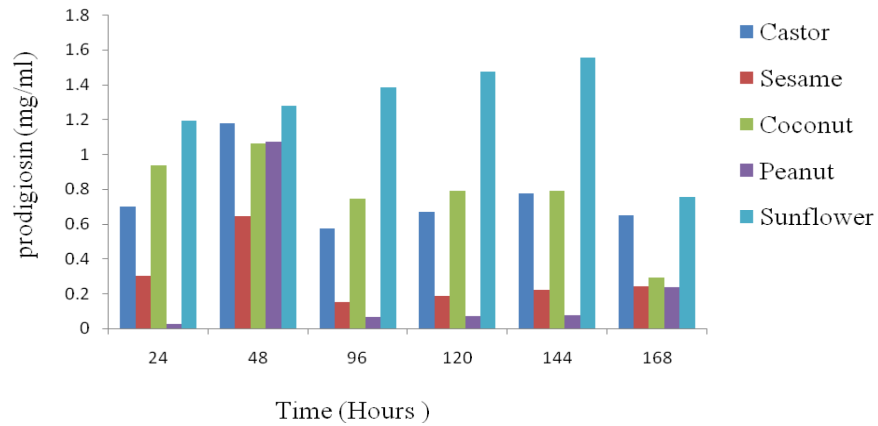


Figure.9 A) Prodigiosin extraction in acetone and B) purification by TLC

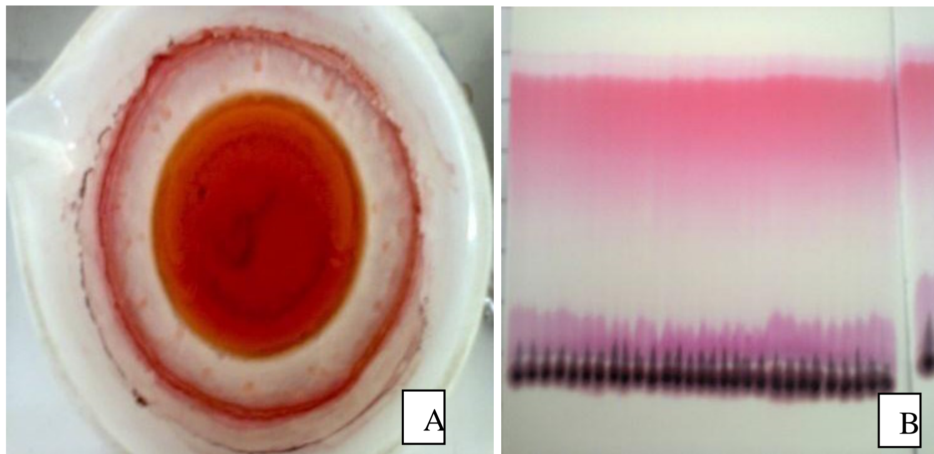
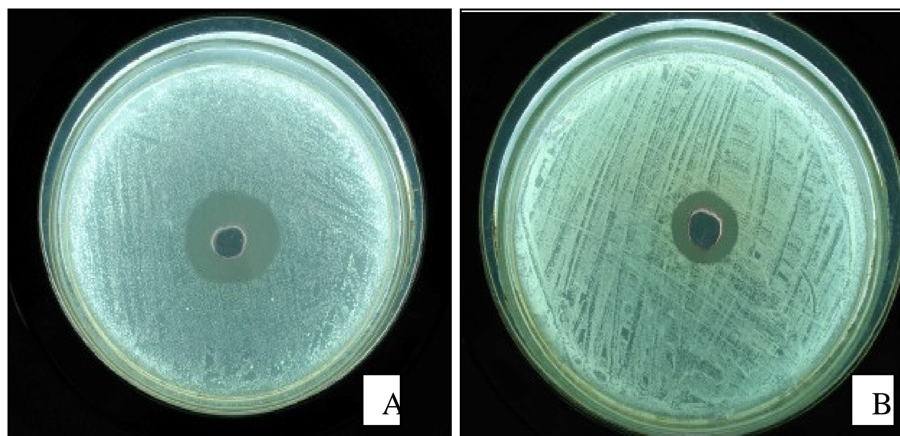


Figure.10 Antibacterial activity of Prodigiosin against A) *S. aureus* and B) *E. coli*



The powdered peanut medium and sunflower seed medium gave the highest yield over all the other media compared in this work. In this work we have compared

nutrient broth with powdered sesame, peanut, castor, coconut and sunflower seed medium. An enhanced pigment production was seen at 28°C in all the different media studied. The yield of prodigiosin from the powdered peanut, sesame, castor and sunflower tested at 37°C was similar yield in the nutrient broth at 30°C. In nutrient broth the prodigiosin production was completely blocked at 37°C similar to the report of Pryce and Terry (Pryce and Terry 2000). Thus it can be concluded that, temperature has impact in blocking prodigiosin production which varies with media of different substrate compositions. Prodigiosin is previously reported to be produced maximally at pH 7. We here report highest yield of prodigiosin at pH 6 in sunflower seed broth supplemented with amino acids and maltose. Also use of castor as carbon source in medium for prodigiosin production is described for the first time in our study. Antibacterial activity results indicated that the prodigiosin antibacterial activity was higher against Gram positive bacteria as compared with Gram negative bacteria. The results showed that prodigiosin is a powerful agent in eliminating plasmid of *P. stuartii.* Since prodigiosin is an intercalating agent, it can cause complete inhibition of plasmid replication. Blood agar lysis method was included in this study since it is widely used to screen for biosurfactant production. The results obtained indicate *Serratia marcescens*as good bioemulsifier. Purified prodigiosin also showed good anticancer potential but no immunosuppressive potential.

Acknowledgement

We express our deep sense of gratitude to University Grants Commission (UGC) for funding this project and to Department of Microbiology as well as to the Principal of Modern College of Arts, Science and Commerce, Shivajinagar, Pune 5 for providing space and infrastructure to carry out this research.

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