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OPTIMIZATION OF CULTURAL CONDITIONS FOR THE PRODUCTION OF PRODIGIOSIN BY *SERRATIA MARCESCENS* AND SCREENING FOR THE ANTIMICROBIAL ACTIVITY OF PRODIGIOSIN

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ABSTARCT

Prodigiosin are a group of natural pigments produced by *Serratia marcescens.* The pigment is produced by various bacteria like *Pseudomonas magneslorubra, Vibrio psychroerythrous, Streptomyces spectabilis, etc.,* This pigment is known for its anti bacterial, antifungal, antiprotozoan and anticancer activity. The present investigation focuses on the optimization of cultural parameters like incubation period, pH, oils, cultural media, and carbon and nitrogen sources, to enhance the production of Prodigiosin from *S.marcescens*, followed by its chromatographic analysis.

KEY WORDS: ***Serratia marcescens,*** **optimization, antimicrobial activity.**

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INTRODUCTION

Natural products either synthesized or secreted by organisms represent one of the critical sources of potential medicinal use. One of these smaller molecular weight natural products secreted by organisms and are having no demonstrable function on the secreting cells are known as secondary metabolite. These secondary metabolites include pigments, enzymes, steroids, and antibiotics. These products are being widely used recently for therapeutic treatment. Among the secondary metabolites, biopigments are obtained from two sources namely the plants (Papageorgiou et al 1972) and microorganisms (2). Biopigments produced from microorganisms are preferred over those from plants because of their stability (3) and availability for cultivation throughout the year (4, 5) One of the classic examples of the biopigment obtained from microorganisms is prodigiosin and prodigiosin like pigments produced by various gram negative and gram positive bacteria (6). Prodigiosins are the family of natural pigments, characterized by a common pyrrolyl pyrromethane skeleton of low molecular weight, appearing only in later stages of bacterial growth. It has been shown to be associated with extracellular granules (7, 8). It has been discovered that prodigiosin exhibits antibacterial, antifungal, antidiabetic, non steroidal, anti tumour (9,10), cytotoxic (11), anti protozoal (12) and antiinflammatory properties. It has got wide application in dying wool and silks.

2. MATERIALS AND METHODS

2.1. Isolation and identification of Serratia marcescens

For isolation of *S. marcescens,* soil sample was collected, serially diluted and plated on nutrient agar and incubated at 30°C for 24 hours. Following incubation, the red colored colonies were selected and propagated on the same medium until pure cultures were obtained. The isolate was identified by gram staining, standard biochemical characterization, colony pigmentation according to Bergey's Manual of Determinative Bacteriology 8th edition (Buchanan and Gibbons, 1975) and MALDI-TOF analysis (results not shown). The colonies were further sub cultured and maintained on nutrient agar slants at 4°C.

Identification of the pigment

A loop full of inoculum was aseptically inoculated into 50ml nutrient broth. The flask was kept in a shaker incubator at 120rpm for 18 hours at 30°C. After incubation, the culture broth was centrifuged at 10,000 rpm for 10 minutes at room temperature. The supernatant was discarded and the pellet at the base of the centrifuge tube was suspended in equal amount of 95% of methanol and centrifuged for 10 minutes at 10,000 rpm. The supernatant was then divided into two portions. One part was acidified with a drop of concentrated HCl and the other part was alkalinized with a drop of concentrated ammonia solution. A red or pink color in the acidified solution and a yellow or tan color in the alkaline solution indicate a positive presumptive test for Prodigiosin (13).

2.2. Optimization of parameters for enhancing prodigiosin production

2.2.1. Media for pigment production

For optimization of the media for enhancing the yield of prodigiosin, 1ml of overnight culture of *S. marsacens* was inoculated into tryptone broth, LB broth, nutrient broth and glycerol broth. The culture flasks were incubated at 30°C in shaker incubator and the rate of pigment production was estimated at intervals of 24, 48, 72 and 96 hours.

2.2.2. Effect of incubation period of prodigiosin production

One ml of overnight culture of *S. marsacens* was inoculated into 50ml glycerol broth and incubated at 30°C in a shaker incubator. The pigment production was estimated at intervals of 24, 48, 72 and 96 hours.

2.2.3. Effect of sugar substrates for prodigiosin production

Carbon source such as fructose, maltose, dextrose, galactose, lactose and sucrose were supplemented at 1% w/v concentration in glycerol broth at 30°C. 1ml of overnight culture of *S. marsacens* was inoculated into the media supplemented with different carbon source. The prodigiosin production was estimated at intervals of 24, 48, 72 and 96 hours.

2.2.4. Effect of pH on prodigiosin production

One ml of overnight culture of *S. marsacens* was inoculated into glycerol broth media maintained at pH 4, 5, 6, 7, 8 and 9. The flasks were incubated at 30°C. Prodigiosin production was estimated after 10, 24, 48, 72 and 96 hours of incubation period from each flask.

2.2.5. Effect of nitrogen source on pigment production

One ml of overnight culture of *S.marsacens* was inoculated into glycerol broth supplemented with 0.5% w/v of nitrogen sources viz., urea, tryptone, peptone, beef extract powder and yeast extract powder; incubated at 30°C for 96 hours. The prodigiosin production was estimated after intervals of 24, 48, 72 and 96 hours.

2.2.6. Effect of oil substrate for prodigiosin production

Oils such as gingelly oil, coconut oil, neem oil, kardi seed oil were supplemented in glycerol broth at 1% w/v. One ml of overnight culture of *S.marsacens* was inoculated into each flask. Culture flasks were incubated at 30°C. Pigment production was estimated after 24, 48, 72 and 96 hours.

3. Extraction of pigment

For extraction of the pigment from the bacterial cells, the culture broth was centrifuged at 10,000rpm for 10 minutes. The supernatant was discarded and the pellet was suspended in equal volume of 95% of methanol and centrifuged again at 10000 rpm for 10 minutes. The pellet was transferred to a fresh plate (14).

4. Estimation of prodigiosin

The maximum absorption spectrum of the pigment was determined in the range of 300-800 nm using UV-Visible spectrophotometer (Thermo scientific, Genesis 10s UV-vis). The concentration of prodigiosin was determined by spectrophotometric analysis, employing a standard graph of known concentration of Prodigiosin (15).

5. Chromatographic Separation

In order to characterize the pigment, thin layer chromatography (TLC) was performed. The methanol extracted pigment of 7-8µl was loaded on a silica gel plate and then placed in the solvent chamber containing methanol, ethylacetate and chloroform in the ratio of 6:3:1.

6. Antimicrobial activity of Prodigiosin

Antimicrobial activity of Prodigiosin was studied against different Gram positive and Gram negative bacterial pathogens like *Vibrio species, Streptococcus epidermidis, Pseudomonas aeruginosa, Escherichia coli, Stapylococcus aureus*, and clinical fungal isolates like *Candida albicans* and *Candida neoformans* by disc-diffusion technique. Sterile discs (10 mm) were individually impregnated with different concentrations (20, 30, 40, 50 and 60µl) of crude methanolic extract of prodigiosin. 95% of methanol was taken as a control and a standard antibiotic (ampicillin). All the discs were placed on the surface of the test bacterial and fungal lawn. Following incubation for 18 to 24 hours at 37°C the plates were examined for the zones of inhibition.

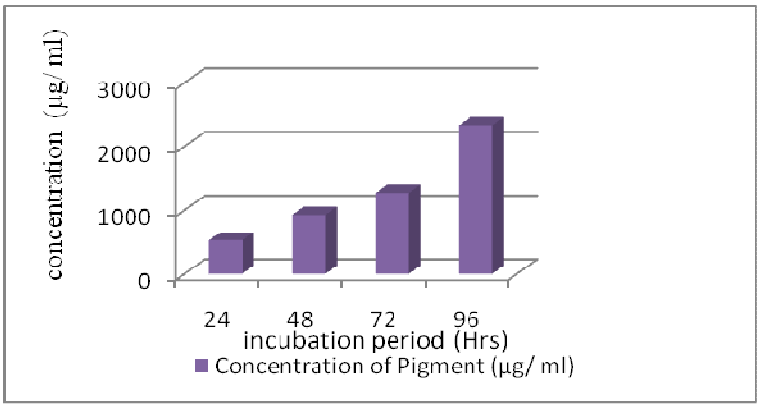
7. RESULTS AND DISCUSSION

The pigment producing strain isolated from soil sample was identified as *S. marcescens.* A red or pink color in the acidified solution and a yellow or tan color in the alkaline solution indicate a positive presumptive test for Prodigiosin (13). The pigment extracted from the isolated *S. marcescens*, turned red in the acidified solution and yellow in the alkaline solution indicating a positive presumptive test

for Prodigiosin. The maximal pigment absorbance was read at 499nm.

Effect of incubation period on prodigiosin production

The culture was observed at different incubation period like 24, 48, 72 and 96 hours. The result showed maximum pigment production at the 96th hour of incubation (16). Prodigiosin production was found to commence after 24 hours of incubation and its production increased with the increase in the incubation period (Graph 1).

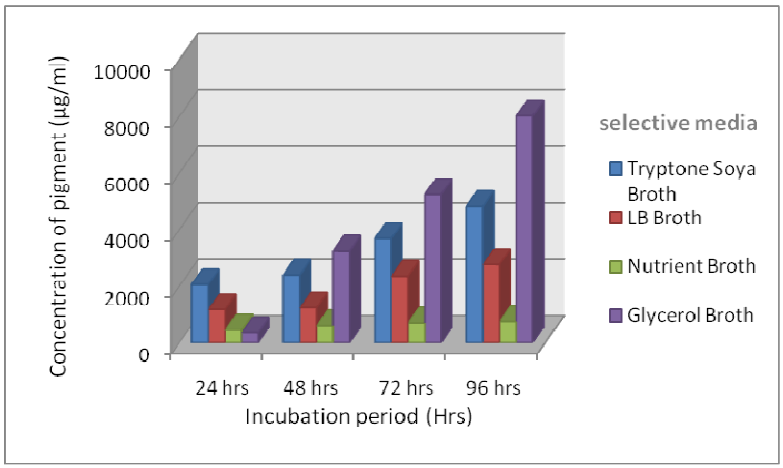


Graph 1

Influence of incubation period on prodigiosin production

Effect of synthetic media on prodigiosin production

Glycerol broth was found to be suitable medium for prodigiosin production (8010 µg/ml after 96 hours of incubation). Followed by tryptone soy broth, LB broth and nutrient broth. The rate of pigment production increased after every 24 hours of incubation (Graph 2).



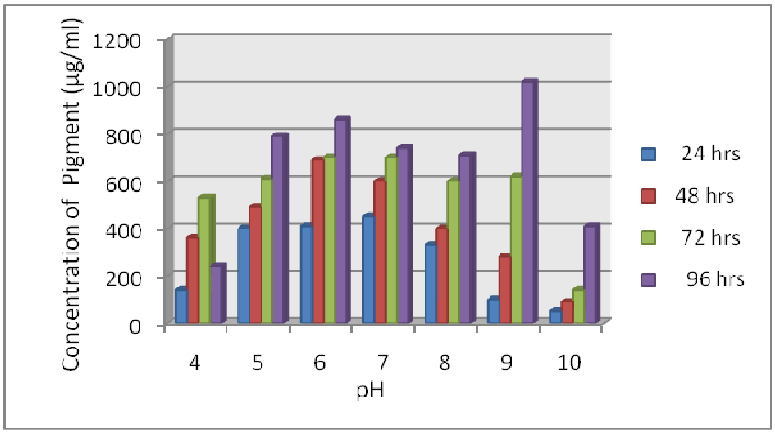
Graph 2

Influence of incubation period on prodigosin production

This result is in perfect agreement with the previous results were enhanced pigment production was observed in the glycerol broth at 30˚C over nutrient broth (17). Hejazi and Falkiner (18) also reported the highest increase in biomass and maximal pigmentation in cultures grown in glycerol medium.

Effect of pH on prodigiosin production

The maximal prodigiosin production was obtained at pH 7. The result of the present study correlates with the observation of Sundaramoorthy et al., 2009 who reported the maximum production of prodigiosin at pH 7. At pH of 4, 5, 9 and 10 a delay in the rate of pigment production was observed and there was a gradual increase in the rate of pigment production after every 24 hours of incubation (Graph 3).



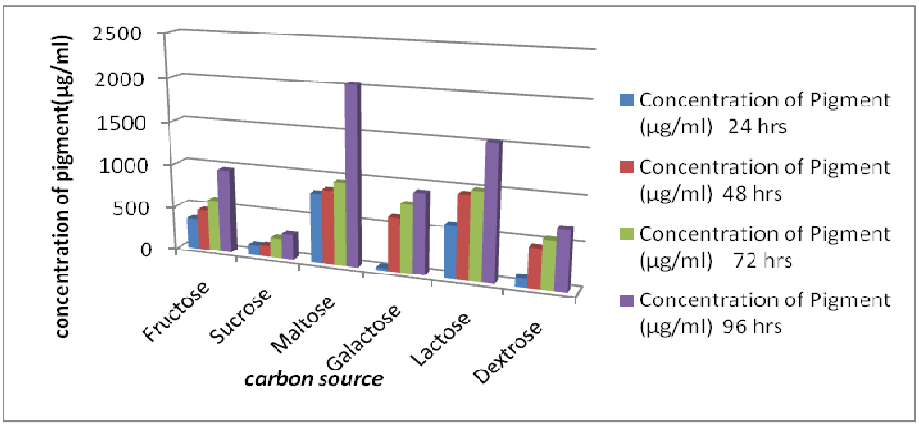
Graph 3

Influence of pH on Prodigiosin production

A complete block in prodigiosin production was observed in most of the basically used media at basic pH (19). The effect of pH value on the pigment productivity can be attributed to two reasons, the first is the effect on the properties of the culture medium including the solubility of the nutrients, transport and ionization, and the second is the effect of pH on the stability of the pigment.

Effect of different carbon sources on prodigiosin production

Nutrient broth amended with maltose favoured the highest production of prodigiosin that increased every 24 hours of incubation. After 96 hours of incubation, the concentration of prodigiosin was found to be 2040 µg/ml spectrophotometrically (Graph 4).



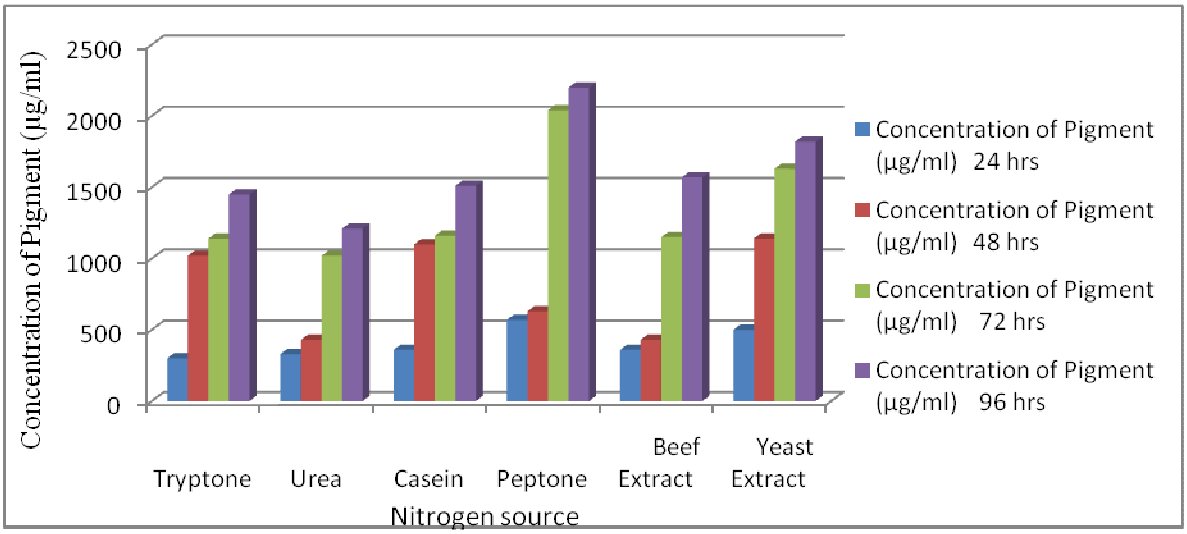
Graph 4

Effect of different carbon sources on prodigiosin production.

The role of glucose when incorporated in the media is critical. Our study revealed that glucose when incorporated in the media resulted in the decreasing pigment concentration which may be due to a repressive effect on the prodigiosin synthesis pathway.

Effect of different nitrogen sources on prodigiosin production

Organic nitrogen source peptone supported the maximum pigment production at 96 hours of incubation. Media supplemented with urea showed least prodigiosin production (Graph 5).



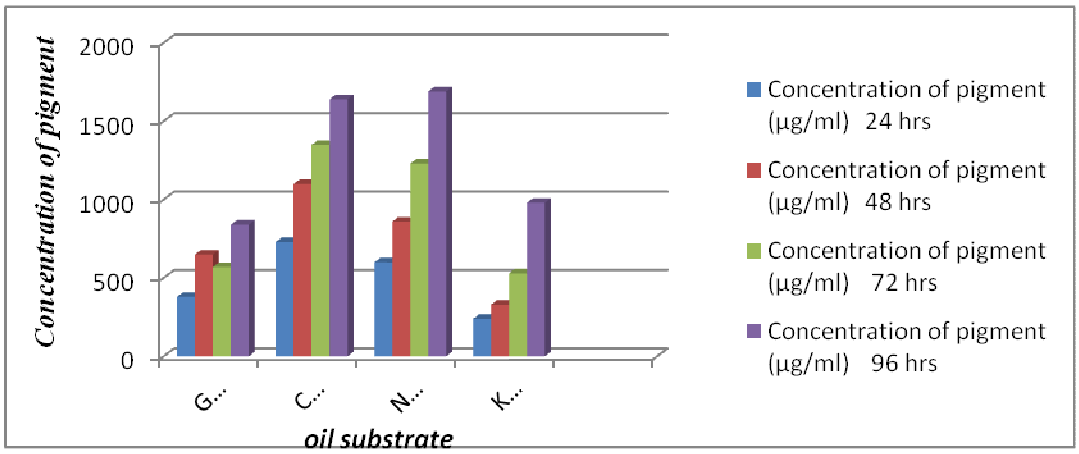
Graph 5

Influence of Nitrogen sources on Prodigiosin production

In the present study, among the various nitrogen sources employed, peptone was found to enhance the growth rate of *S. marcescens* and thereby increased the production of prodigiosin. At an interval of 24 hrs, an increase in prodigiosin production was observed. Maximum production of 2200µg/ml was observed after 24 hours. There was a delay in the rate of production of the pigment in the media supplemented with urea and it may be due to the alkaline conditions of the environment due to the release of ammonium compounds. This suggests that ammonium salts or the inorganic nitrogen sources have inhibitory activity on the production of prodigiosin. This fact is in accordance with the result obtained by Hejazi and Falkiner (18) who found out that *S. marcescens* grown in the mineral media did not produce pigment when the carbon source was glucose and the nitrogen source was urea, ammonium acetate and ammonium sulphate.

Effect of Oil Substrates on prodigiosin production

Neem oil showed a highest rate of pigment production at 96 hours of incubation, which was followed by the media containing coconut oil as substrate. Kardi seed oil and sesame oil was responsible for moderate pigment production. The media amended with mustard oil as substrate shows least pigment production. And after 48 hours of incubation it gradually declined (Graph 6).

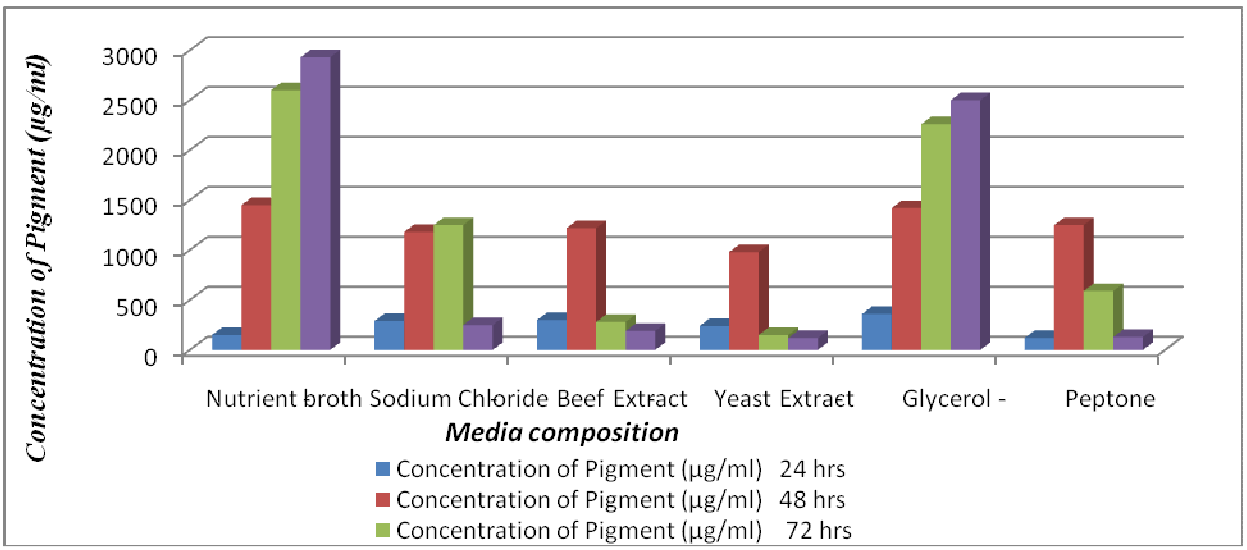


Graph 6
Influence of oil substrates on prodigiosin production

The oils are known for their high levels of unsaturated fatty acid content and very low percentage of saturated fatty acids which were studied and reported by Giri et al 2004. The observed enhancement in the yield of prodigosin has been attributed to the high saturated fatty acid content. From the arguments it was speculated that bonded fatty acids are of limited carbon source and less accessible by *S. marscescens*.

Effect of media composition on a prodigiosin production

A study regarding the effect of media composition on the production of prodigiosin revealed that *S. marcescens* proliferated and produced maximum pigment in the media supplemented with glycerol. In the media devoid of sodium chloride, beef extract, yeast extract and peptone the production of prodigiosin declined after 48 hours of incubation. Nutrient broth was used as the control to optimize the parameter.



Graph 7
Influence of media composition on prodigiosin production

Thin Layer Chromatography

Three different fractions, characterized by purple, pink and orange bands with R_f of 0.19, 0.71 and 0.85 respectively were obtained. When the methanolic extract of prodigiosin was run using a solvent system composed of methanol, ethylacetate and chloroform in the ratio of 6:3:1. The results are shown in table 1.This results were in accordance with the investigations carried out by Gulani et al (20).

Table 1
Rf values of different fraction of methanolic extract of prodigiosin

Methanolic Fractions of prodigiosin	Rf value
Purple	0.19
Pink	0.71
Orange	0.85

The orange band at R_f 0.85 had only an evanescent orange colour which rapidly turns red on exposure to air (Husain et al., 2004). The blue coloured band with R_f 0.19 was present consistently. The pink band at R_f 0.71 was always present, but only in very small amounts (21).

Antimicrobial activity

The antimicrobial activity was performed by disc diffusion revealed a greater inhibition towards both Gram positive and Gram negative bacteria and pathogenic fungi. The results are shown in table 3 which proved that with the increase in the concentration of prodigiosin (20, 30, 40, 50 and 60µl) there is an increase in the antimicrobial activity of prodigiosin which is exactly correlating with the results of Khanafaeri et al (22). The methanol was used as the control and it does not exhibit the antimicrobial property. This study confirms the potential of prodigiosin as an effective antimicrobial agent and the results are tabulated in table 2.

Table 2
Antimicrobial activity of prodigiosin

Sl.No	Name of the organism	Diameter of Zone of inhibition (in mm)						
		Standard Antibiotic (amp)	Control (methanol)	Methanolic extract of prodigiosin				
				20µl	30µl	40 µl	50 µl	60 µl
1	*Vibrio species*	25mcg	40 µl	_	1 ± 01	2 ± .03	3 ± .00	6 ± .02
2	*Streptococcus epidermidis*	25mcg	40 µl	2 ± .00	2 ± .01	5 ± .02	7 ± .02	13 ± 02
3	*Pseudomonas aeruginosa*	25mcg	40 µl	_	_	1 ± .06	10 ± 02	16 ± 04
4	*E. coli*	25mcg	40 µl	2 ± .01	2 ± .03	4 ± .01	8 ± .03	10 ± .04
5	*Staphylococcus aureus*	25mcg	40 µl	2 ± .03	3 ± .00	3 ± .03	5 ± .02	8 ± .03
6	*Candida albicans*	Amphotericin -B	40 µl	_	1 ±. 04	14 ± .02	20 ± .01	23 ± .04
7	*C. neoformans*	Amphotericin -B	40 µl	_	_	8 ± .04	11 ± .0	14 ± .08

(-) Absence of detectable zone of inhibition
All experiments were conducted on triplicates and the results were expressed as mean ± S.E.M

CONCLUSION

The red pigment produced by *Serratia marcesens* was isolated and characterized. Methanol was found to be an ideal solvent for the maximal extraction of the membrane bound pigment . The pigment recorded maximum absorption at 499 nm.Thin Layer chromatography shows three different band which are to be analysed and studied in future The present study reveals that prodigiosin is an effective antimicrobial agent against Gram positive and Gram negative bacteria and also for human fungal pathogens. We sincerely thank everyone for helping us in completing the work.

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