



Production, Partial Purification and Antimicrobial Activity of Red Pigment by *Serratia marcescens*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Serratia marcescens are the secondary metabolite for production of red pigment. This pigment is a promising drug owing having antifungal, immunosuppressive and anti-proliferative activity. In given study optimization of LB for maximum yield of pigment production was performed. The pigment produced was partially purified to study its antimicrobial activity. The optimization for pigment production was carried out to increase pigment production using parameters viz; Incubation period, pH, Temperature, Percent inoculum, Oil substrates play a vital role in pigment production. LB media was supplemented with Sesame, Groundnut, Coconut, and Sunflower oil to enhance pigment production. The purity of pigment was checked by TLC and SGC. The partially purified form of the pigment showed antimicrobial activity against *Staphylococcus aureus*.

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1. INTRODUCTION

Microorganisms have been used for a long time for the production of diverse molecules like antibiotics, enzymes, vitamins, texturizing agents, pigments and so on. The toxicity problems caused by those of synthetic origin pigments to the environment have created a mounting interest towards natural pigments. Among natural pigments, pigments from microbial sources are potentially good alternative to synthetic pigments. Natural pigments can be obtained from two natural sources viz; plants and microorganisms. Pigments from plants have many drawbacks like instability against heat, light, pH, insolubility in water and nonavailability throughout the year (Venil, 2009).

The advantages of pigments from microorganisms include easy and fast growth by using cheap culture media, independence from weather conditions and colors of different shades. Many artificial synthetic colorants which have been widely used in food stuff, dye stuff cosmetic and pharmaceutical manufacturing processes comprise various hazardous effects. To counter the ill effects of synthetic colorants, there is worldwide interest in process development for the production of different pigments from natural sources (James, et al., 2000, Morohoshi, et al., 2007).

Microorganisms produce various pigments like carotenoids, melanins, flavins, quinones, prodigiosins, and more specifically monascins, violacein or indigo. Microbial colors are used in fish industry to enhance the pink color of farmed salmon (Venil, 2009). Some natural colorants are used commercially as antioxidants. Carotenoids such as xanthophyll and β carotene like astaxanthin play a major role in the metabolism of eye macula and retina and in maintaining good vision. β carotene play a role in the prevention of cancer. Other xanthophylls like adinorubin and astaxanthin act as nutraceuticals that prevent carcinogenesis through antioxidative, anti free radicals or other mechanisms. The beneficial nutraceutical functions of the carotenes and xanthophylls extend to the prevention of heart attack and stroke (Venil, 2009). *Monascus species* fungi which produce monascus pigments has been used for the production of traditional East Asian Foods such as red rice wine, red bean curd (Mohan, et al., 2009). Monascorubrin and monascoavin are orange and yellow

pigments isolated from *Monascus purpureus mentii*. They are widely used for coloring food stuffs. *Cryptococcus neoformans* produce a melanin pigment that plays a important antioxidant funtion with melanised cryptococcal cells being more resistant to oxygen and nitrogen derived oxidants than nonmelanised cells. Production of pigment by Group B *Streptococcus* also confers resistance to oxidative stresses including H_2O_2 and superoxide (Venil, 2009). The phenazine pigments pyocyanin and pyorubrin from *Pseudomonas aeruginosa* has antimicrobial activity and are used as food coloring agents (Saha, et al., 2008).

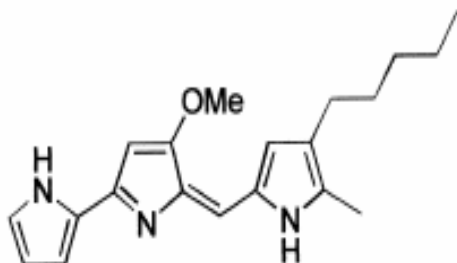
The aim of the present study was to develop a simple and economical medium for red pigment production by *Serratia marcescens* and to study its antimicrobial activity.

Serratia marcescens is an aerobic, facultative anaerobic, motile, spore forming, enteric saprophytic rod shaped bacteria (Raj, et al., 2009, Khanafari, et al., 2006). Gram negative bacteria of the serratia are oppurtinistic human, plant and insect pathogens and are members of *Enterobacteriaceae*. It can grow in temperatures ranging from 5°C - 40°C and in pH ranging from 5 to 9. *Serratia marcescens* has been isolated from soil, water, air, food stuff, plant surface and animals. It produces a spectrum of virulence factors including chitinases, proteases, lipases and nuclease which are capable of damaging human cells and tissues. It can cause infections of the respiratory tract, urinary tract, wounds, blood stream and nosocomial infections that are clinically problematic (Mekhael, et al., 2008).

Prodigiosins are red pigmented family naturally occuring (tripyrrol methane structure) linear tripyrole ring. Prodigiosin is a characteristic member of a group of compounds with a common pyrrolylpyrro methene (PPM) skeleton (4 methoxy, 2-2 bipyrole ring) and has a series of close relatives bearing the same PPM core with different alkyl substituents (Wei, 2005). Other variants of prodigiosin are undecylprodigiosin, cycloprodigiosin, metacycloprodigiosin, dipyrrolydipyrromethane which depends on different organisms (Venil, 2009). These are emerging broad spectrum of compounds having distinct biological activities like antibacterial, antifungal, antiprotozoal, cytotoxic, antitumor, anticancer, antimalarial, immunosuppressive, antidiabetes, antioxidants, non steroidal

antiinflammatory drugs, dyeing of silks and wools (Raj, et al., 2009, Montaner, et al., 2000, Kavitha, et al., 2010, Mekhael, et al., 2008, Han, et al., 2001)

Serratia marcescens and other species of *Serratia* majorly produce prodigiosin or its derivatives. The structure of prodigiosin is given below.



Pic 1. The structure of prodigiosin

Prodigiosin is produced by *Serratia marcescens*, *Pseudomonas magnesorubra*, *Vibrio psychroerythrus* (Aoust, et al., 1974). *S. rubidaea*, *Vibrio gazogens*, *Alteromonas rubra*, *Rugamonas rubra*. Gram positive actinomycetes such as *Streptovercillium rubriterculi* and *Streptomyces longisporus ruber* form prodigiosin and derivatives of this molecule (Khanafari, et al., 2006, Slater, et al., 2003).

2. MATERIALS AND METHODS

Given work were deals with to develop a simple and economical medium for the red pigment production by *Serratia marcescens* for antimicrobial activity.

Optimization of media

The study aimed to optimize the media used in the production of pigment from *Serratia marcescens* (MTCC 86) using various materials, media LB and nutrient broth. Oils used like sesame oil, groundnut oil, coconut oil and sunflower oil.

The pigment produced was quantified by taking the ratio at 534 nm/615nm. The quantify the pigment from remaining tubes after an interval of 48, 72, 96, and 120 hours.

The incubation period was optimized by preparing an inoculum of sterile MTCC nutrient broth with a single colony of *Serratia*

marcescens. The culture was transferred to sterile polypropylene tubes and centrifuged at 2500 rpm for 20 minutes. The supernatant was discarded and sterile D/W was added to the cell pellet to make O.D. 0.2 at 615 nm using a colorimeter.

The media is prepared and autoclaved, and 1% of the prepared inoculum was inoculated in LB broth in butt tubes and incubated at 30°C for 24, 48, 72, 96, and 120 hours. The absorbance at 615nm and organism was harvested. The supernatant was discarded and the pellet was re-suspended in acidified ethanol, vortex and centrifuged at 2500 rpm for 20 minutes (Sundaramoorthy, et al., 2009, Giri, et al., 1983, Ding, et al., 1983).

Partial purification of pigment

- TLC process involves dissolving crude pigment in acetone in Durham's tube allowing it to dry completely and then placing the sample on a silica gel coated sheet. The solvent is then allowed to run until it reaches about half a centimetre below the top of the plate (Selvameenal, et al., 2009, Song, et al., 2006).
- Silica gel chromatography is used dissolving the pigment in chloroform adding silica powder to create slurry and fixing glass wool at the bottom of the column. The solution of methanol: chloroform: ethyl acetate (5:30:65) is added to elute the sample. The eluted sample fractions are collected in saline tubes and the elution rate is adjusted to get 1ml/min samples in tubes. The TLC of all collected samples was performed using the same solvent. The fractions with good separation were selected and used for antibacterial assay (Venil, 2009).

Screening of antibacterial activity: purified pigment was tested using Mueller Hinton agar, agar powder, and nutrient broth. Antibiotic standard (Ampicillin 10 ug/ml), McFarland's solution (BaCl_2 , H_2SO_4), and partially purified pigment were used. The McFarland's turbidity assay was performed by preparing a 0.5 solution by mixing 0.05ml of 1% BaCl_2 and 9.95 ml of 1% H_2SO_4 . The O.D. at 570 and the colonies of the bacteria were inoculated under aseptic conditions. The O.D. of all bacterial suspensions was matched with the standard McFarland's solution (Harley, et al., 2005).

Well Diffusion Method uses the previously prepared pigment solution at 14mg/ml and 10mg/ml concentrations. Wells were prepared using a sterile cork borer and 30 ul of each

sample was loaded in each well. The plates were kept in an incubator at 37°C for 16 to 18 hours, and the zone of inhibition was observed (NCCLS, 1999, Collee, et al., 2005).

3. RESULTS AND DISCUSSION

3.1 For Media Optimisation



Fig. 1. Pigment production in LB broth



Fig. 2. Pigment production in LB with oil

Optimization of different parameters was studied to obtain maximum yield of pigment. The results obtained for optimization are as follows.

Table 1. Results for incubation period optimization

Sr.No.	Hours	Abs at 615 nm	Abs at 534 nm	Ratio (534nm/615nm)
1	24	0.29	0.640	2.206
2	48	0.46	1.329	2.889
3	72	0.58	1.233	2.125
4	96	0.62	2.626	4.235
5	120	0.58	2.394	4.127

Maximum pigment production was obtained after 96 hours

Table 2. Results for pH optimization

Sr.No	pH	Abs at 615 nm	Abs at 534 nm	Ratio (534 nm/615 nm)
1	5	0.93	1.106	1.189
2	6	0.88	1.576	1.790
3	7	0.67	1.575	2.350
4	8	0.66	1.200	1.790
5	9	0.47	0.986	2.09

Maximum pigment production was seen at pH 7

Table 3. Results for temperature optimization

Sr.No	Temperature	Abs at 615nm	Abs at 534nm	Ratio (534nm/615nm)
1	RT	0.37	0.908	2.454
2	25°C	0.38	0.992	2.610
3	30°C	0.57	2.242	3.933
4	37°C	0.27	0.556	2.059

Maximum pigment production was seen at 30°C.

Table 4. Results for LB media supplemented with different oils

Sr.No	Oil	Abs at 615nm
1	LB	0.31
2	Groundnut	1.05
3	Sunflower	1.07
4	Sesame	1.18
5	Coconut	1.32

Maximum cell density was seen by using coconut oil

Table 5. Results for percentage of coconut oil in LB

Sr.No	Percent	Abs at 615 nm
1	0.5%	1.73
2	1%	1.90
3	2%	1.78
4	3%	1.76
5	4%	1.75
6	5%	1.70

Maximum cell density was seen by using 1% coconut oil

Table 6. Results for percent inoculum optimization

Sr.No	Percent inoculum	Abs at 615 nm	Abs at 534 nm	Ratio (534nm/615nm)
1	0.5%	0.40	1.155	2.510
2	1.5%	0.40	1.026	2.565
3	2%	0.41	0.976	2.380
4	2.5%	0.58	1.680	2.896
5	3%	0.35	0.435	1.242

Maximum pigment production was seen by using 2.5% inoculum.

3.2 Chromatography

a) Thin layer chromatography

The separation of crude pigment was performed using different solvents.

n hexane: chloroform
(50:50)

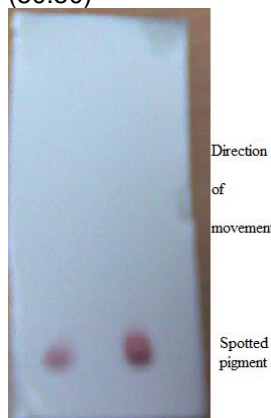


Fig. 3. TLC using n hexane: chloroform

n butanol: acetic acid:
water (60:30:10)

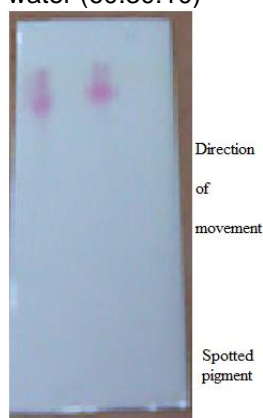


Fig. 4. TLC using n butanol: acetic acid: water

Methanol: chloroform:
ethyl acetate (5:30:65)

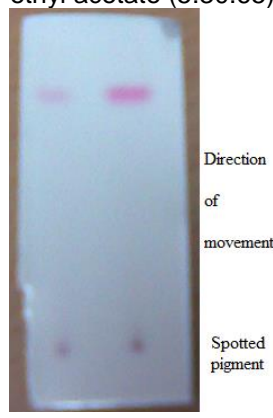


Fig. 5. TLC using methanol: chloroform ethyl acetate

n hexane: ethyl
acetate (3:1)



Fig. 6. TLC using n hexane: ethyl acetate

Table 7. Results for Thin Layer Chromatography

Sr. No	Solvent	Rf value
1	n hexane: chloroform (50:50)	No separation
2	n butanol: acetic acid: water (60:30:10)	0.882
3	Methanol: chloroform: ethyl acetate (5:30:65)	0.821
4	n hexane: ethyl acetate (3:1)	No proper separation

Methanol: chloroform: ethyl acetate (5: 30: 65) showed better pigment separation and hence was selected as the solvent for further processes.

b) Silica gel chromatography

Silica gel chromatography was performed and the fractions were eluted. The eluted fractions were as follows:

Table 8. Results for partial purification of pigment by Silica Gel chromatography

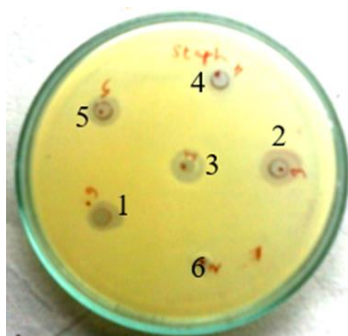
Sr. No	Fractions	Color
1	1-10	Colorless
2	11-21	Dark pink
3	22-28	Light pink

TLC of all fractions was performed using methanol: chloroform: ethyl acetate (5: 30: 65).

- Fractions from 1-10 no pigment present.
- Fractions from 11-21 good separation with single bands.
- Fractions from 22-28 impurities with improper separation.

3.3 For Antimicrobial Activity

The results of antibacterial activity of the partially purified pigment against *Staphylococcus aureus* are as follows

**Fig. 7. Plate showing antimicrobial activity of partially purified pigment against *Staphylococcus aureus*****Table 9. Results for antimicrobial activity of partially purified pigment**

Sr. No.	Test Microorganisms	ZONE OF INHIBITION (Diameter in mm)					
		Pigment (14 mg/ml)	Pigment (10mg/ml)	Pigment+ Amp (14 mg/ml+ 10ug/ml)	Pigment+ Amp (10 mg/ml+ 10ug/ml)	PC (10 ug/ml)	NC
1	<i>Escherichia coli</i> (ATCC 25922)	-	-	<10	<10	-	-
2	<i>Staphylococcus aureus</i> (ATCC 25923)	12	10	11	10	13	-

PC: Positive control: Ampicillin: 10ug/ml
 NC: Negative control: DMSO, Amp: Ampicillin

Serratia marcescens has been grown on different types of media for the maximum production of pigment. It was studied already in more details and pigment was generally recognized as Prodigiosin or Prodigiosin like pigments. These pigments show many biological activities like antibacterial, antifungal, antitumor, immunosuppressive etc. In this project, we have focused our study for optimization of different growth parameters for maximum yield of red pigment. We also focused our study for partial purification of pigment to check its antimicrobial activity (Akin-Osanaiye, et al., 2019).

Media like nutrient broth, soybean casein digest, oil seed broths, brain heart infusion medium etc. have been used for the pigment production. LB medium along with oils has also been used. So, in our present study we have focused on standardizing LB by using different parameters like incubation period, pH, temperature, percent inoculum and various oils.

Comparatively higher pigment production was obtained after 96 hours at pH 7 at 30°C by using 2.5% inoculum. Among the different oils used 1% coconut oil enhanced the pigment production. So, it is quite possible to obtain bulk production of pigment by using coconut oil which is readily available and cheap.

The crude pigment obtained was checked for its antimicrobial activity at concentrations like 100ug/ml, 250ug/ml, 500ug/ml, 750ug/ml, and 1mg/ml. But it did not show activity against *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923). Further, crude pigment was checked for its purity by Thin Layer Chromatography. Methanol: Chloroform: Ethyl acetate (5: 30: 65) was selected as the good solvent. Silica gel chromatography yielded partially purified pigment.

Pigment production was enhanced using 1% coconut oil. The pigment obtained from media with oil was also purified by silica gel chromatography. But the oil could not be separated from the pigment, so the concentration of purified pigment could not be estimated. This form of pigment also showed antimicrobial activity against *Staphylococcus aureus*. It did not show any activity against *Escherichia coli*.

The partially purified form of the pigment was checked for its antimicrobial activity at various concentrations viz; 100ug/ml, 250 ug/ml, 500ug/ml, 1mg/ml and further concentrations. But no activity was seen. We have tried to check

the antimicrobial activity of the partially purified pigment alone and its synergistic action along with Ampicillin against *Staphylococcus aureus* and *Escherichia coli*. Zone of inhibition was seen at very high concentrations of pigment. No synergistic action was seen. Enhanced activity might be seen if higher concentrations will be used for crude pigment or partially purified pigment. There is also need to purify the pigment in more pure form.

In future work, can be undertaken to enhance pigment production by using coconut oil and separate the oil from pigment. It can also be used to check activities like immunosuppressive, antitumor and cytotoxicity and as antimalarial drug.

4. CONCLUSION

The present project was centered on formulating a production medium for effective production of pigment and to study its antimicrobial activity.

The results obtained showed that Luria Bertani (LB) medium gave good yield of pigment after 96 hours at pH 7 at 30°C by using 2.5% inoculum. Production was enhanced using 1% coconut oil.

Partial purification of crude pigment was done using TLC and silica gel chromatography. This purified form showed antimicrobial activity against *Staphylococcus aureus* at high concentration. No results were obtained for *E. coli*.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Authors hereby declare that no generative AI technologies such as large language models (Chat GPT, COPILOT etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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