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ISOLATION, IDENTIFICATION OPTIMIZATION OF PRODIGIOSIN PIGMENT PRODUCED BY *Serratia marcescens.*

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ABSTRACT

Some microorganisms can produce variety of biopigments which is involved in colouring purpose. In this study isolation and identification of *Serratia marcescens* was done with suitable methods. Prodigiosin pigment was extracted from *Serratia marcescens*. To optimize various physico-chemical conditions for maximum pigment production was done. Antimicrobial, antioxidant activity and preparation of red colour nail paint and candle was performed by using prodigiosin pigment which showed and excellent result. This pigment can be used as a colouring agent instead of using chemical dyes.

KEYWORDS

Serratia marcescens, Prodigiosin pigment, Optimization, TLC, Antimicrobial activity, Antioxidant activity, Nail paint and Candle.

INTRODUCTION

Color is one of the significant visual properties and is an important attribute of any article. The color determines the acceptance of a product and has paramount influence on human life. Many synthetic colors used in foodstuff, dyestuff, cosmetics and pharmaceutical manufacturing pose various hazardous effects like allergies, tumor, cancer and severe damages to the vital organs [1]. Moreover, the effluent of synthetic dyes poses serious threat to the environment. Consequently, many synthetic colors have been banned due to their toxicological problems. With the increasing awareness about the toxic effects of synthetic colors and consumer safety, there is an increasing interest in the development of colors from natural sources [2].

Natural colors are generally extracted from fruits, vegetables, roots and microorganisms which are often called as bio-colors due to their biological origin. The utilization of natural pigments in foodstuff, dyestuff, cosmetics and pharmaceutical manufacturing processes has increased in the recent years due to their nontoxic nature [3]. Moreover, their eco-friendly, antioxidant, anticancer and antimicrobial activities further add to their positive effects. The significant growth in the naturally derived colors has been attributed to their stability and consumer acceptance. Further the

annual growth rate of naturally derived colors has been predicted to be 5-10 per cent in comparison to synthetic colors, with a low growth rate of 3-5 percent [4].

Although there are a number of natural pigments, only a few are available in sufficient quantities to be useful for industry because they are usually extracted from plants. In spite of the availability of variety of pigments from fruits and vegetables, there is an ever growing interest in microbial pigments due to several reasons like their natural character and safety to use, production being independent of seasons and geographical conditions, controllable and predictable yield [5&6]. In the last few years scientific research has focused itself on studying detailed attributes of not only potentially pathogenic microorganisms, but has also thrown light on a whole new approach to studying them. The rapid growth of microbes reduces the production time to a matter of days compared to plant and animal sources, the production is flexible and can easily be controlled [7].

The presence of biopigments has been reported in almost all the classes of microorganisms including bacteria, fungi, yeasts and algae. These microorganisms can produce variety of biopigments such as carotenoids, melanins, flavones, quinines, prodigiosin, and monascins [8&9]. Many types of yeast like *Rhodotorula* (pink), *Yarrowialipolytica* (brown), *Cryptococcus* (red) and *Phaffiarhodozyma* (carotenoids) are good source of microbial pigments. The pigment production by molds of Monascus group especially *Monascus purpureus* and *Monascus anka* for use as a good food grade color is well known [10].

The algae which produce pigments are *Chlorococcum*, *Chlamydomonas*, *Chlorella*, *Hematococcusi* and *Sporangium.* Another algae namely, *Dunaliella salina* belonging to class chlorophylaceae occur in marine environment and produces β-carotene which can be used as food colorant [7]. The pigment produced by algae and fungi may be less accessible for exploitation because of the structural complexity of the pigment bearing tissues and the pigment production at critical points of development within a complex lifecycle. Bacteria are good source of pigments. Bacterial pigment production is one of the emerging fields of research to demonstrate its potential for various industrial applications [11]. Some pigment producing bacteria are *Staphylococcus aureus* for golden yellow, *Serratia marcesans* for red, *Micrococcus lutes* for yellow, *Micrococcus roseus* for pink, *Staphylococcus roseus* for red and *Pseudomonas cynxanatha* for yellow pigment [12].

Extreme environments generally characterized by a typical temperature, pH, salinity, toxicity and radiation level are inhabited by various microorganisms. The hot springs and geothermal vents are found in different parts of the world which contain several prokaryotes, especially adapted to grow in these environments. These microorganisms are often colored due to the presence of

photosynthetic and carotenoids pigments. The color stability under extreme temperature, variable pH, and processing conditions is the pre requisite for industrial application. Therefore, microbial diversity has been a great source for exploration for application of biopigments. Limited research studies have been conducted for exploration of such pigment-producing microorganisms under Indian conditions [13].

In the present study the *Serratia marcescens* was isolated from the soil sample. Prodigiosin pigment was extracted with suitable methods and its antimicrobial, antioxidant activity and preparation of red colour candle and nail paint was performed.

MATERIALS AND METHODS

1. Collection of Soil Samples

10gm of Rhizosphere soil samples were collected from the depth of 10-15cms at different sites in Bangalore (Chikka banaswadi and Hebbal) using sterile equipment.

2. Isolation of Bacteria

1gm of soil sample were serially diluted and 0.1ml of diluted samples from each dilution was spread over the Nutrient agar surface. Then the plates were incubated at 37°C for 24 hours.

3. Identification of Isolates was performed by Cappuccino Sherman [14].

3.1 Gram's staining were performed to find out strain for gram positive or gram negative.

3.2 Biochemical Characterization of Isolates

Bacterial physiology differs from one species to the other. These differences in carbohydrate metabolism, protein metabolism, fat metabolism, production of certain enzymes, ability to utilize a particular compound etc. help them to be identified by the biochemical tests.

3.2.1 IMViC TEST [14]

The following test was performed accoding to Cappuccino Sherman [14] to identify the members of Enterobacteriaceae family. Indole production test, Methyl Red test, Voges-Proskauer test, Citrate utilization test, Catalase test, Oxidase test, Casein hydrolysis test, Triple sugar iron test and Urease test.

3.3 Optimization for enhancing prodigiosin production was performed by Srimathi [15].

(i) Effect of incubation period

From the overnight culture of the isolates, 1ml was inoculated in nutrient broth and incubated in a shaker incubator at room temperature. The pigment production was estimated at time intervals of 24, 48, 72 and 96 hours.

(ii) Effect of sugar substrates

The carbon sources such as glucose, fructose, lactose, sucrose were supplemented at 1% w/v concentration in nutrient broth at room temperature. Overnight culture of the isolates was inoculated into the media supplemented with different carbon source. The pigment production was estimated at time intervals of 24, 48, 72, and 96 hours.

(iii) Effect of pH

1ml of overnight culture of theisolates was inoculated in to nutrient broth maintained at pH 6, 7, 8 and 9 .The flasks were incubated at room temperature. Prodigiosin production was estimated after 24, 48, 72 and 96 hours of incubation period from each flask.

(iv) Effect of temperature

The effect of temperature on prodigiosin production was observed by inoculating the bacteria in isolates in nutrient broth and incubated at different temperatures such as 25°C, 30°C, 35°C, 40°C.

(v) Effect of nitrogen source

1ml of the overnight culture was inoculated in nutrient broth. The broth was supplement with 1.0%w/v of nitrogen sources like urea, ammonium sulphate, sodium nitrate, sodium nitrite and incubated at room temperature. After 48 hours of incubation prodigiosin production was estimated.

3.4 Extraction of the prodigiosin pigment

Bacterial cultures from the liquid broth were centrifuged at 10,000 rpm for 15 minutes. The supernatant and cell pellet were extracted with acetone and ethyl acetate. Then the cell pellet was repeatedly centrifuged to obtain white pellet. The pigment extracts of ethyl acetate fraction and acetone fraction were evaporated separately in evaporating dishes at room temperature till the powder appears.

3.5 Purification of prodigiosin pigment by Thin Layer Chromatography

Thin layer chromatography is a technique used to separate nonvolatile mixtures. The prodigiosin pigment was separated using TLC plate coated with silica gel. In the chromatographic tank the developing solvent such as methanol, ethyl acetate, chloroform (6:3:1v/v) was standardized and poured, then it was saturated with a filter paper soaking in the mobile phase. Then the Rf value of the chromatogram was observed in the TLC plates.

3.6 Applications of prodigiosin pigment

(a) Antibacterial activity [15]

Antibacterial activity of prodigiosin pigment was studied against different species of Gram positive bacteria such as *Staphylococcus aureus*, *Bacillus* spp. and Gram negative bacteria such

as *Escherichia coli*, *Klebsiella pneumonia* and *Proteus*spp. The test cultures were swabbed on MullerHinton agar plates and activity of prodigiosin was studied using well diffusion method.

(b) Antifungal activity [15]

The antifungal activity of prodigiosin was checked against different species of fungal pathogens including *Aspergillus niger*, *Candia albicans*. The cultures were swabbed on potato dextrose agar plates and the activity of prodigiosin was checked by well diffusion method.

(c)Antioxidant activity [16]

0.05 mM solution of DPPH in methanol was prepared. This solution was added to an equal volume (2 ml) of the solution of the tested compound (dissolved in methanol). Methanol was used as control solution. After 20 and 60 min at room temperature, the absorbance was recorded at 517 nm and compared with the appropriate standard, namely ascorbic acid. Lower absorbance of the reaction mixture indicates higher DPPH free radical scavenging activity. The percentage of scavenging activity of compound on DPPH radical was calculated as percentage (%) inhibition of DPPH (I %) using the following formula:

$$TAC(\%) = (As-Ac) / (Aaa-Ac) * 100$$

(Ac=Control, As=Sample, Aaa=Amino acid)

(d) Nail paint [17]

Nail paint was prepared by purchasing transparent color nail paint and then 2 ml of the prodigiosin was added into it. Stability testing and microbiological analysis of the cosmetics were carried out.

(e) Candle [17]

Candles were made by melting wax in a container. The mould was then greased with oil and the melted wax was poured into the mould and 1mL of prodigiosin was added. One control candle was prepared which does not contain the pigment.

RESULTS AND DISCUSSION

1. Characteristics of the Soil Sample:

This study revealed that ten Rhizosphere soil samples were analyzed with respect to bacteria, where as *Serratia marcescens* was the most prevalent species. The physiochemical properties of soil play an important role in the growth of microorganism. The Rhizosphere soil was slightly acidic.

2. Isolation and Identification of Bacteria from soil samples:

The collected soil samples consists large number of different groups of pigment producing bacteria with different morphology and individual colonies were picked up separately & purified by Quadrant streaking in nutrient agar plates. The preliminary identification of the bacterial isolates revealed the presence of *Serratia marcescens* and confirmed with morphology and biochemical Characterization (Table 1 & 2).

Table:1 Morphological characteristics of *Serratia marcescens*

Culture isolate	Configuration	Elevation	Surface	Pigment	Opacity	Gram staining	Cell shape
	Round	Convex	Smooth	Pink	Opaque	Gram Negative rod	Rod

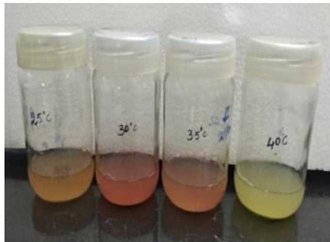
Table: 2 Biochemical characteristics of *Serratia marcescens*

Premilinary and Biochemical Tests	*Serratia marcescens*
Gram staining	Gram negative rod
Motility	Motile
Catalase	Positive
Oxidase	Negative
Indole	Negative
Methyl red	Negative
Voges –proskauer	Positive
Citrate Utilization Test	Positive
Triple Sugar Iron Test	Positive
Starch hydrolysis	Negative
Casein hydrolysis	Positive
Urease Test	Positive

3. Optimization for enhancing prodigiosin production

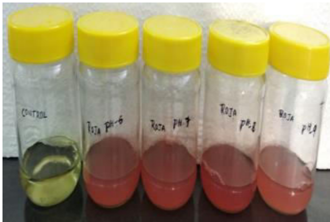
3.1 Effect of incubation period:

The OD value of the cultures was observed using spectrophotometer at different incubation period like 24, 48, 72 and 96 hours. The results showed maximum pigment production at the 96th hour of incubation. Prodigiosin production was found to commence after 24 hours of incubation and its production increased with the increase in the incubation period.



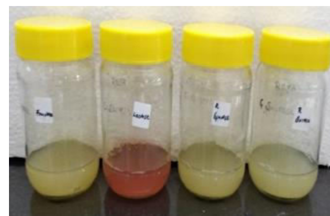
3.2 Effect of temperature:

The highest yield of prodigiosin pigment was observed at 30ºC followed by 25ºC, 30ºC, 35ºC, and 40°C.



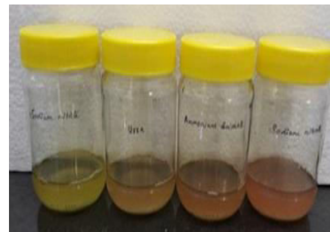
3.3 Effect of pH:

The pH affects the production of prodigosin pigment. The maximum production of prodigiosin pigment is at pH 8.



3.4 Effect of sugar substrates

Nutrient broth amended with lactose favoured in the highest production of prodigiosin pigment that increased every 24 hours of incubation.



3.5 Effect of nitrogen source

Ammonium sulphate supported the maximum pigment production and the yields increased prodigiosin pigment.

Table 3. Optimization for enhancing prodigiosin production

Temperature	OD at 540nm	pH	OD at 540nm	Carbon Source	OD at 540nm	Nitrogen Source	OD at 540nm
25°C	0.06	6	0.90	Glucose	0.87	Ammonium Sulphate	0.33
30°C	0.83	7	0.96	Sucrose	0.89	Urea	0.29
35°C	0.63	8	1.04	Fructose	0.90	Sodium Nitrate	0.26
40°C	0.72	9	0.87	Lactose	0.91	Sodium Nirtite	0.20

4. Production of pigment in nutrient broth

In the present study nutrient broth was used for the production of the pigment, the yield of prodigiosin pigment on nutrient broth was observed only after 96 hours of incubation.

5. Estimation of prodigiosin pigment

The results were studied after 24, 48, 72 and 96 hours time intervals. The bacterial cell absorption prior to pigment extraction as noted at every step. The prodigiosin pigment was estimated using the following formula

Prodigiosin unit/cell = [OD499 – (1.381x OD620)] x1000 / OD

OD= optical density; OD499=Pigment absorbance; OD 620=Bacterial cell absorbance;

1.381 =constant.

6. **Extraction of the prodigiosin pigment**

The pellet was extracted with acetone which yielded a residual crude pigment. Thus acetone was found to be best for the extraction of pigment from the pellet. Separation of supernatant and collection of crude extract was done.

7**. Purification of prodigiosin pigment by Thin Layer Chromatography**

In the present study, the pigment purification was done by using thin layer chromatography.

After purification, the RF value of this pigment was 0.78.

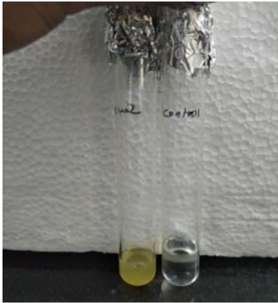
8. Applications of prodigiosin

1. Antibacterial activity

Antibacterial activity of prodigiosin pigment extracted from *Serratia marcescens* was assayed against seven bacterial strains such as *Staphylococcus aureus, Salmonella* spp., *Escherichia coli, Klebsiella pnemoniae, Enterococcus faceae, S. haemolyticus, Pseudomonas aeruginosa*. From this assay, *Staphylococcus aureus* and *Escherichia coli* showed highest zone of inhibition.

2. **Antifungal activity**

Antifungal activity of the prodigiosin pigment was assayed against *Aspergillus flavus* and *Candida albicans*. From these *Candida albicans* showed highest zone of inhibition.



3. Antioxidant activity

Prodigiosin pigment extracted from selected strain shows antioxidant activity by reducing the DPPH radical. DPPH is a stable radical (violet colored) and when it reacts with an antioxidant compound which can donate hydrogen or electron, it is reduced to yellow colored diphenylpicrylhyfrazine. The % value of inhibition of DPPH (1%) for pigment prodigiosin is 32% prodigiosin pigment also showed antioxidant activity.

4. Candle

Prodigiosin pigment was in plain wax and successfully color the candle; wax without pigment was kept as a control.

5. Nail paint

Nail paint was prepared using prodigiosin. Stability testing and morphological analysis were carried.

DISCUSSION

In the present study the pigment prodigiosin produced by the bacterium S*erratia marcescens* has many applications such as dyeing, antibacterial, antifungal activity and antioxidant activity. Frequently, prodigiosin was done in nutrient broth, Nutrient broth of peptone, yeast extract as the major components. Peptone is a commercially existing digest of plant or animal protein made accessible to organisms as peptides and amino acids to aid the requirement for sulphur, nitrogen, carbon and energy.

Peptone does not contain some minerals and vitamins. Yeast and meat extracts contain eukaryotic tissues which are extracted by boiling and then concerted to powdered form. Nutrient broth is used for the pigment production as it is basically devoid of carbon sources; it is also associated with yeast extract and maltose.

The chief producer of prodigiosin pigment is *Serratia marcescens* and in this production carbon source may well play a critical role. There is a proof representing that the bacterium *Serratia marcescens* grow well on artificial media using different compounds as a sole carbon source. It was reported that the bacterium produced higher amount of prodigiosin at 30°C and at pH 8. Cerdeno and Furstner reported that prodigiosin pigment is a natural compound, it has antifungal, antibacterial, algicidal, antiprotozoal, antimalarial, cytotoxic, anticancer and antiproliferative properties [18&19]. Darah suggested that the bacterial cells need an alkaline condition to produce the antibacterial activity. Prodigiosin has antibacterial activity as it inhibits various species of gram positive and gram negative bacteria. Mordants are applied on the textile fabrics to gain altering colours and to enhance the dye uptake, to get better the colour fastness performance of several natural dyes [20].

CONCLUSION

Based on the present study, an attempt was carried out to isolate the pigment producing *Serratia marcescens* from the soil samples. The red pigment producing bacteria was isolated and characterised using nutrient broth. The pigment was dyed on wax and transparent nail paint which exposed good colour tone. The pigment also has antimicrobial activity and antioxidant activity. In large scale production, the pigment will make it an alternate to chemical dyes.

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