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Production and Extraction of Bacterial Pigments from Novel Strains And Their Applications.

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ABSTRACT

Bacterial pigments have various applications in current scenario. Pigmented bacteria otherwise called as chromo bacteria, mostly aerobic producethe pigments. Our present study is aimed at isolating the bacterial pigments from the four strains such as *Bacillus subtilis, Enterococcus hirae, Acinetobactor mufti, Pseudomonas aeruginosa*. Among these *Pseudomonas aeruginosa* produces various colored pigments and maximum pigment production was observed at optimum temperature of 37^{o}C at 72hrs. The extractwere prepared by chloroform extraction method. The samples were analyzed by using UV spectrophotometer and HPLC. Anti-oxidant activity study using DPPH assay revealed the presence of 54.7% antioxidants. The pigments were evaluated for their potential as food colorants with 3% agar. The pleasant coloration was observed at 500µg/mL.Hemolytic activity of the pigments inferred that, nohemolysis observed with both pigments. Antibacterial activities of the pigments wereevaluated against pathogenic bacteria maximum growth inhibitory activity was observed against *citrobactor sp.*

Keywords: Aerobic bacteria, *Pseudomonas aeruginosa*, Chloroform Extraction, DPPH, Food colorant, HPLC

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INTRODUCTION

Color is the most pleasing attribute of any article. Blue or green color suggests cool and peaceful environment and encourages relaxation. The color is associated with quality and sensory properties of the food. The first characteristic perceived by our senses is appearance or color of food, which not only determines its appearance or color of food, which not only determines its acceptance, but even helps in its recognition. A well-textured food, rich in nutrients and flavor, cannot be eaten unless it has the right color. Due to the consumer awareness and concern for healthful and perfectly balanced food, increasing interest in the food colorants of natural origin has been developed [1]. Consequently, the consumer wants to see the food looking natural as the synthetic colors has been proved to be carcinogenic to humans. Plants, animals, and microbes are the sources of natural pigments. When the microbial cells are used to produce the color the term refers to 'microbial pigments'. Different types of pigments extracted from different microbes. Microbial pigment production is a recent phenomenon.They display all the colors from rainbow including light or dark tinges and unusual colors like black, white, brown, golden, silver and fluorescent green, yellow or blue.

MATERIALS AND METHODS

Culture collection

The test organisms were collected from department of Biotechnology, Vel Tech High Tech Dr. RR Dr.SR Engineering College Avadi, Chennai. Such organisms are,

Bacillus subtilis, Enterococcus hirae, Acinetobacter mufti, and *Pseudomonas aeruginosa*

Substrate used for fermentation

In our study, nutrient agar and brain heart infusion agar medium used forfermentation medium for pigment production [3].

Screening of pigment producing organism

1 ml of the test organisms such as *Bacillus subtilis, Enterococcus hirae, Acinetobacter mufti, Pseudomonas aeruginosa,* were inoculated into 20ml test tube containing 10ml nutrient broth and brain heart infusion broth separately. The contents were inoculated at 37ºC for 24hours.

Culture conditions

In one set of experiments, 250ml of Erlenmeyer flasks containing 50ml of growth medium were inoculated with a calculated amount of culture and incubated at different temperature such as 15- 50ºC. In another set of experiments, the pH of the medium was varied as 4-11. In both the sets, the flasks were incubated with shaking for 6 days. All shake flask experiments were done in triplicate [3].

Determination of growth

Aliquots (1ml) of the culture, diluted to 10 ml using 25%Nacl solution, were used to measure the groth interms of optical density (O.D) at 660nm with a spectrophotometer. Theexperiments were carried out in triplicate.

Production of microbial pigments

Pseudomonas aeruginosa was grown in the nutrient agar medium at 37ºC for 24hours. Then transfer the culture to the nutrient broth contains (gL^{-1}) peptone-5, Nacl-5, yeast extract-3, pH 7±0.02.the contents were incubated at 35ºC for 72hours in rotary shaker and observed the color change. (Fig-1)

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Figure 1: Production of microbial pigments

Extraction of microbial pigments and analysis by UV spectrophotometer

The broth culture was centrifuged at 10000rpm for 5mins at 4ºC.the pigment was extracted by using chloroform (1:2) to the supernatant and the aqueous phase was discarded.(fig- 2)To the solvent phase 0.2N Hcl was added and the color change was observed [3]. The extracted pigments (fig-3) were evaporated (fig-4) to dryness and redissolved in 0.1ml chloroform. The pigments were analyzed by using UV/Visible spectrophotometer and the absorbance was measured [8].

Figure 2: Extraction of microbial pigments

Figure 3: Extracted microbial pigments

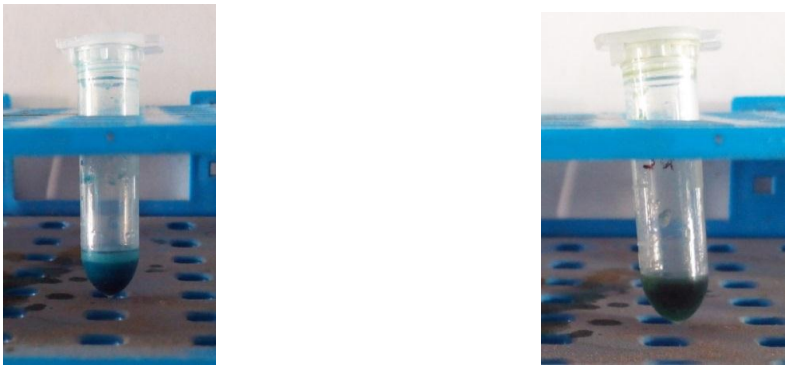

Figure 4: Evaporated microbial pigments

DPPH (1, 1-DIPHENYL-2-PICRYL-HYDRAZIL) free radical scavenging activity

The free radical scavenging activity of the fractions of chloroform extract of microbial pigment was measured by 1, 1-Diphenyl-2-Picryl-Hydrazil (DPPH). The various fractions of microbial pigments (100µg/ml to 500µg/ml) were prepared by using DMSO.1ml of DPPH solution was added to the all fractions (fig-5). Then the absorbance was measured at 517nm by using UV-visible spectrophotometer and the as 1ml of DPPH solution. [6]. DPPH scavenging effect was calculated.

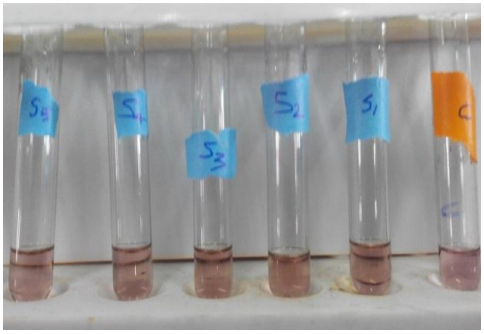

Figure 5: DPPH Free radical scavenging activity of microbial pigments

Pigments as food colorant

Various concentrations of microbial pigments were prepared (100µg/ml to 500µg/ml) and 3% agar was added to the pigment solutions and heated to boiling and cooled to solidification. This test was made to ensure the reliability of the pigments and their coloring ability at boiling temperature in food materials [5].

Antibacterial assay

Antibacterial assay was carried out by the well diffusion technique for each pigment along with a control (chloroform for microbial pigments and distilled water for microbial pigments).wells with 0.5cm diameter is made on sterile nutrient agar plates. Pathogenic bacteria namely salmonella paratyphi, Escherichia coli, citrobacter sp. and klebsiella pneumonia were swabbed on the surface of the agar and different concentration of microbial pigments (100µg/ml to 500µg/ml) were added into the wells. Plates were incubated at 37ºC for 24hours.Antibacterial activity of the microbial pigments was determined by measuring the growth inhibition around the well [8].

Hemolytic activity

Fresh sheep blood was collected from a slaughter house and EDTA (2.7gm in 100ml distilled water) was added as anticoagulant at the rate of 5%of the volume of blood. The blood was centrifuged at 5000rpm for 7mins at 4ºC along with a normal saline and the supernatant was discarded.1ml of the packed RBC thus obtained and it was resuspended in normal saline to obtain a 1%RBC suspension.the same procedure was adopted for chicken blood also.the assay was carried out in Laxbro microtitre plates .the various fractions of microbial pigments were prepared.one row of well used for only one concentration.initially 100µl of normal saline was added to each well. The 100µl of lowest concentration of the microbial pigment was added to the first well and this process was repeated up to the last well from which 100µl was discarded.then 100µl of the prepared erythrocyte suspension was added to each well.a negative control was kept by mixing 100µl of normal saline and 100µl of 1% RBC suspension.formation of a fine button cell with a regular margine indicates the negative reaction. A uniform red coloured suspension of the lysed RBC indicates the positive result.the plates were incubated for 3hours at 37ºC and the hemolysis was observed [5].

HPLC analysis of the pigment

The pigment was analyzed by HPLC (Waters 600) equipped with a UV-Vis detector (Waters 2489). The pigment extracts were filtered through a 0.45 µm hydrophobic PTFE membrane (Waters). Chromatographic separation was performed on a reverse-phase column (C18, 4.6x250 mm, Waters) where the temperature of

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the column was maintained at room temperature and the mobile phase was methanol at a flow rate of 1 ml/min. The pressure was 756 psi and the injection volume was 1 ml [2].

RESULTS AND DISCUSSION

Screening of pigment producing bacteria

The test organisms such as Bacillus subtilis, Bacillus cereus, Acinetobacter mufti, Pseudomonas aeruginosa, Enterococcus hirae grow on the nutrient broth and brain heart infusion broth. (Fig-6(a))The investigated results are presented here. Among those organisms pseudomonas aeruginosa produced microbial pigments. So, it was selected for pigment production.

Culture growth on nutrient agar plates

Pseudomonas aeruginosa was growing on nutrient agar plates. It shows the formation of blue-green colonies. The media contains sterilized nutrient broth (g/L)(peptone-5, Nacl-5, yeast extract-3) along with agar(20g).the pH should maintained for their growth at 7±0.02. And then it was incubated for 24hrs at 37ºC. After incubation, it will be in blue-green colored colonies(fig-6(b)).Then the colonies are transferred to the nutrient broth medium for the pigment production with optimized temperature and pH and media constituents.

(a) ***Bacillus cereus***

(b) ***Pseudomonas aeruginosa***

(c) Bacillus subtilis

(d)*Acinetobacter mufti*

Figure 6(a): screening of pigment producing bacteria

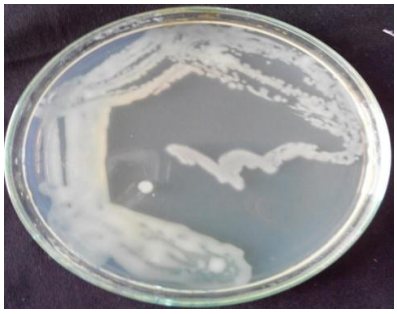

Figure 6(b): ***Pseudomonas aeruginosa*** **on the nutrient agar medium**

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Culture conditions

Temperature, pH and incubation period usually plays an important role in cell synthesis. And suitable temperature, pH and incubation time are needed for culture growth and their pigment productions. Before producing pigments, optimization of above all are important criteria.

Effect of temperature on pigmentation and growth

Fig-7&8, demonstrates the maximum growth and pigment production temperature at 37ºC. There was a gradual and uniform decrease in growth and pigment production.

Effect of pH on the pigmentation and growth

Fig-9&10, demonstrates the maximum growth and pigment production pH at 7.there was a gradual and uniform decrease in growth from pH 7 to 11.

Effect of incubation period on the pigmentation and growth

As shown in fig-11, the culture has a moderate incubation period of 6 days, with along log phase (about 2 days) characterized by an increase in growth. Growth was maximum on day 3, followed by decrease from day 4. Considerable pigment production by the strain was observed after about 24hours of incubation (fig-12), as the culture became dark green color. Pigment production increased to a maximum 5 days of incubation, followed by decrease on day 6. Finally we observed the suitable temperature (35ºC), pH (7), and incubation period (3 days) for pigment production from pseudomonas aeruginosa.

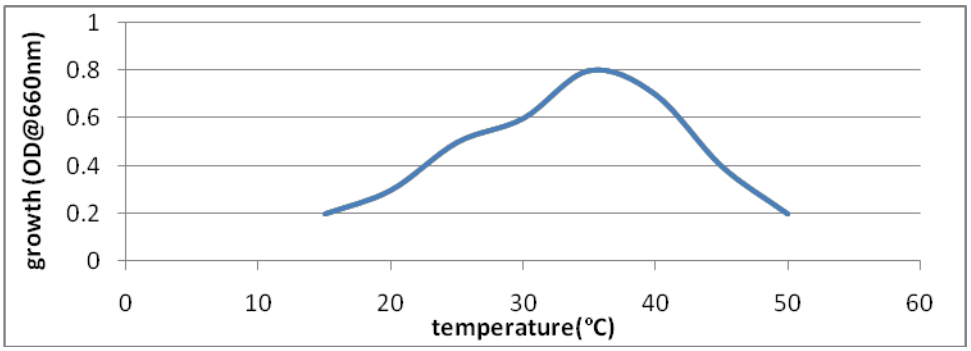

Figure 7: Effect of temperature on growth of *Pseudomonas aeruginosa*

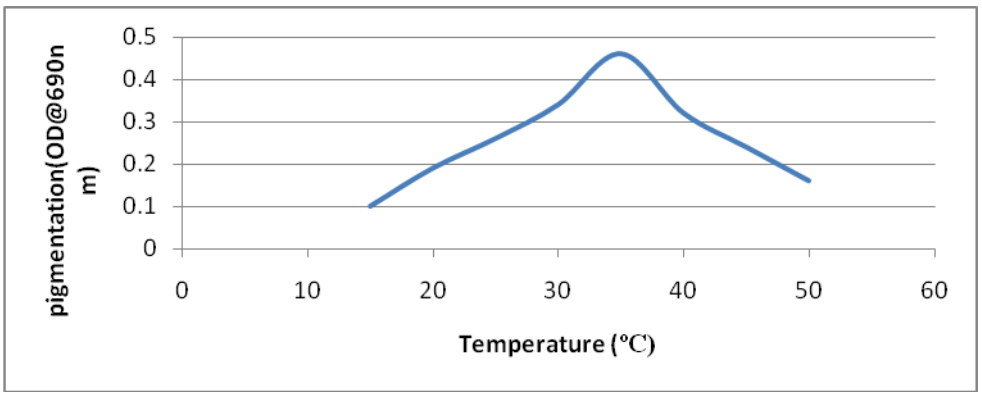

Figure 8: Effect of temperature on pigmentation of *Pseudomonas aeruginosa*

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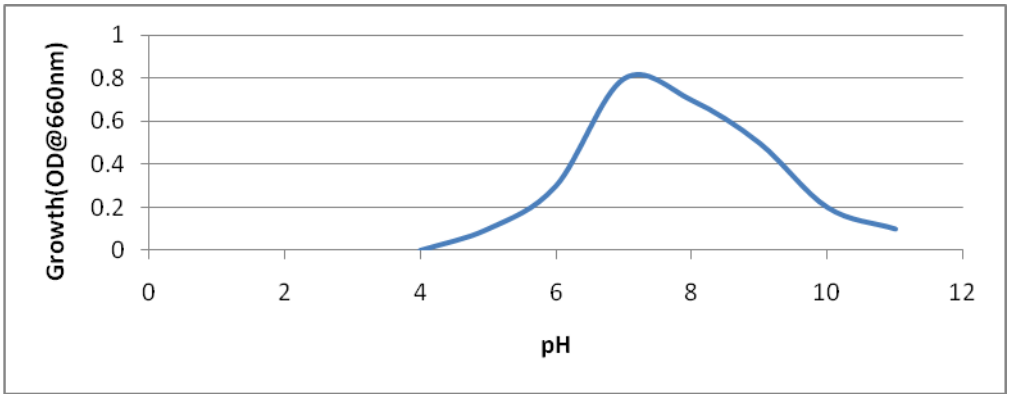

Figure 9: Effect of pH on growth of*Pseudomonas aeruginosa*

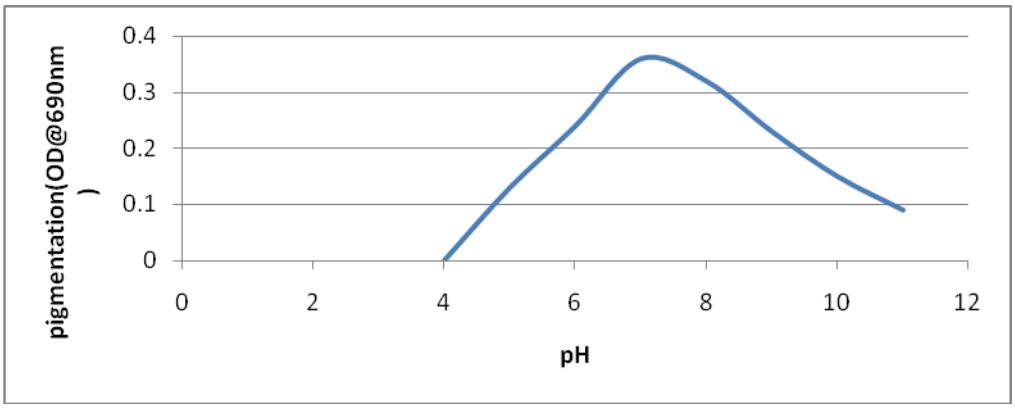

Figure 10: Effect of pH on pigmentation of*Pseudomonas aeruginosa*

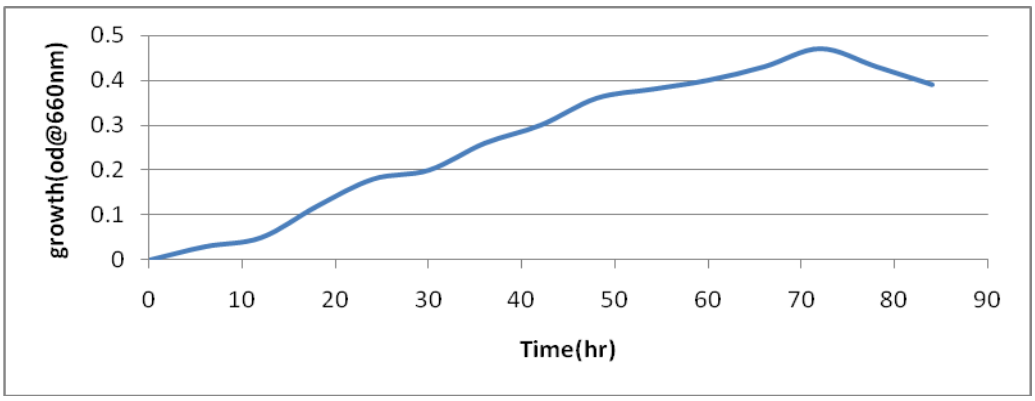

Figure 11: Effect of incubation time on growth of the *Pseudomonas aeruginosa*

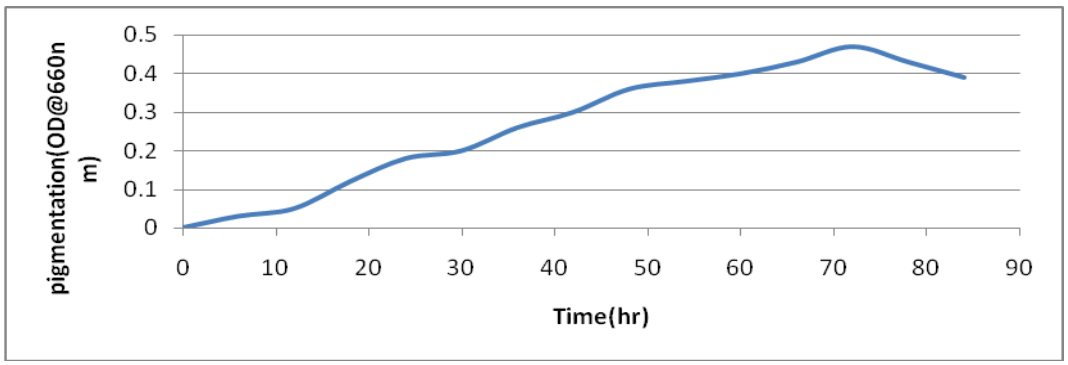

Figure 12: Effect of incubation time on pigmentation of the *Pseudomonas aeruginosa*

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Characterization of spectral analysis

The evaporated pigments was dissolved using chloroform. Blue color pigment was obtained, when extract by chloroform and then blue color was changed into green color. Maximum absorbance values of blue and green color pigments obtained at 690nm and 682nm (fig-13&14).

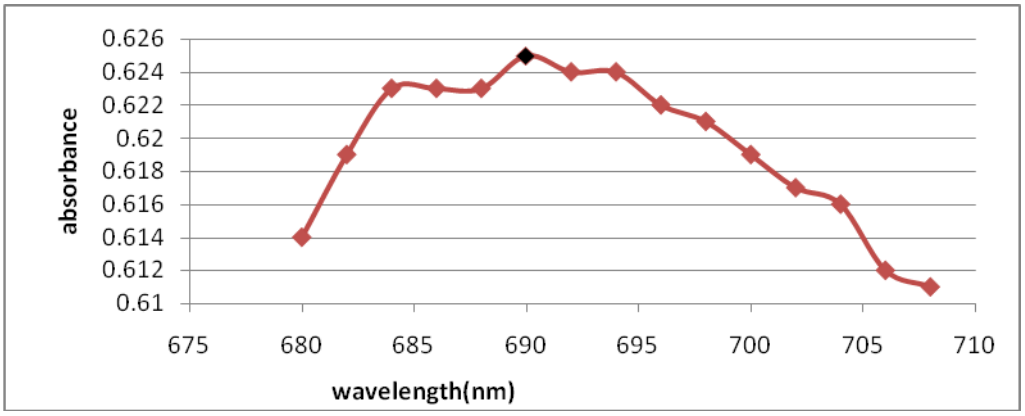

Figure 13: UV-Visible absorption of blue color pigment

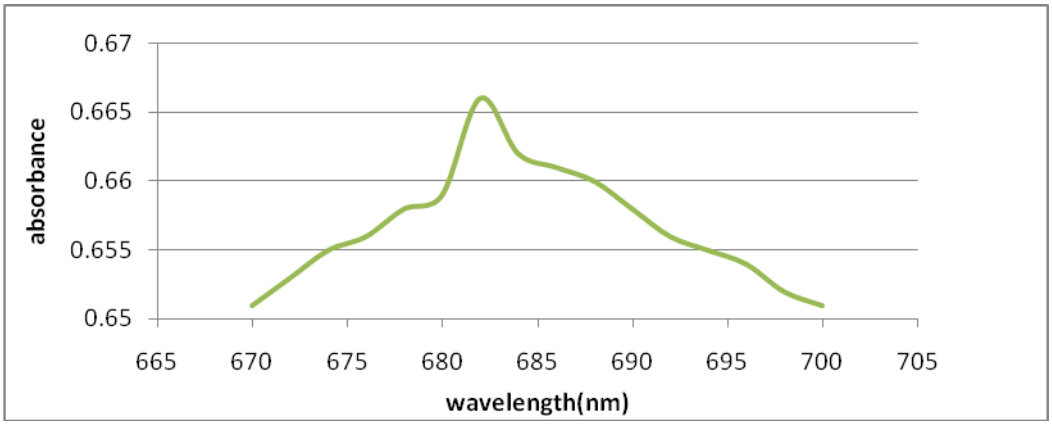

Figure 14: UV-Visible absorption of green color pigment

Free radical scavenging activity

DPPH is a stable radical and is frequently used for evaluating antioxidant activity of natural food colorant products. Fig-15 shows the antioxidant activity of the microbial pigments.

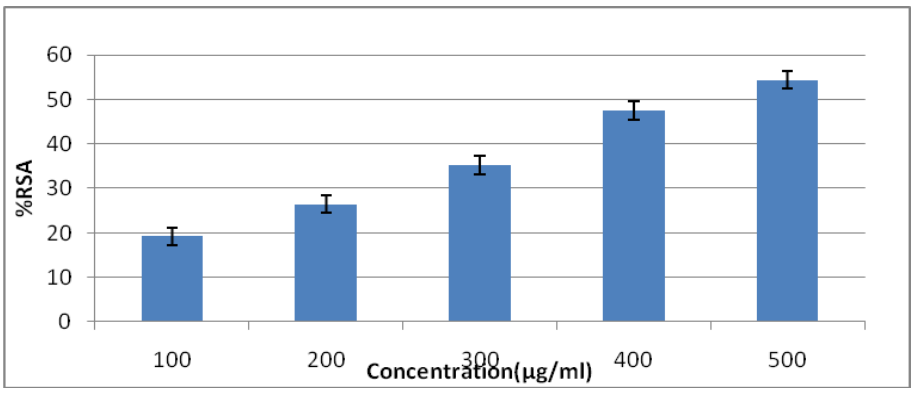

Figure 15: DPPH free radical scavenging activity of pigment from pseudomonas aeruginosa (Error bars represents ± standard deviation (SD))

Food colorant activity

All the concentrations of microbial pigments show the pleasant coloration. The maximum intensity was observed in 500µg/ml (fig-16) and the least coloration was observed in 100µg/ml. even in the lower concentration the color obtained with the pigments pleasant to see.

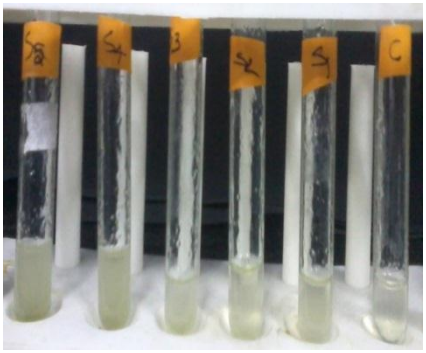

Figure 16: Food colorant activity assay

Antibacterial activity

Pigment extracts from Pseudomonas aeruginosa showed distinct antibacterial activity against citrobacter sp. among the four pathogenic bacteria. Antibacterial activity was found in all the concentrations (100µg/ml to 400µg/ml). Lower concentrations of pigments showed lower inhibitory zone when compared to the higher concentration.

Hemolytic assay

Pigments were found to be hemolytic positive for the concentration of 30, 40 and 50mg/ml in chicken blood. There was no hemolytic activity at 10 and 20mg/ml concentration of microbial pigments. There was no hemolytic activity was observed even in higher concentration i.e., 50mg/ml.

HPLC Analysis of pigments

Fig-17 & 18 shows the HPLC chromatogram of the pigment produced by the isolated bacterium. The pigment probably belongs to the chlorophyll family and pyocyanin.

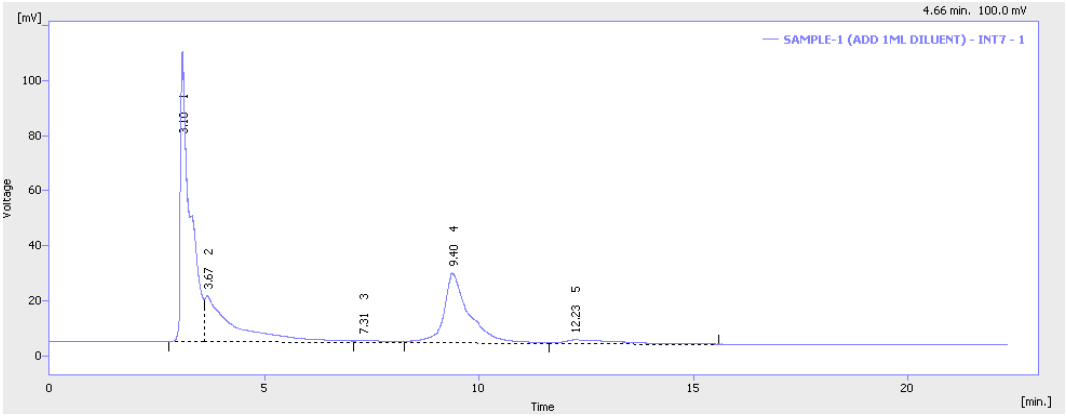

Figure 17: HPLC Chromatogram of blue colored pigments.

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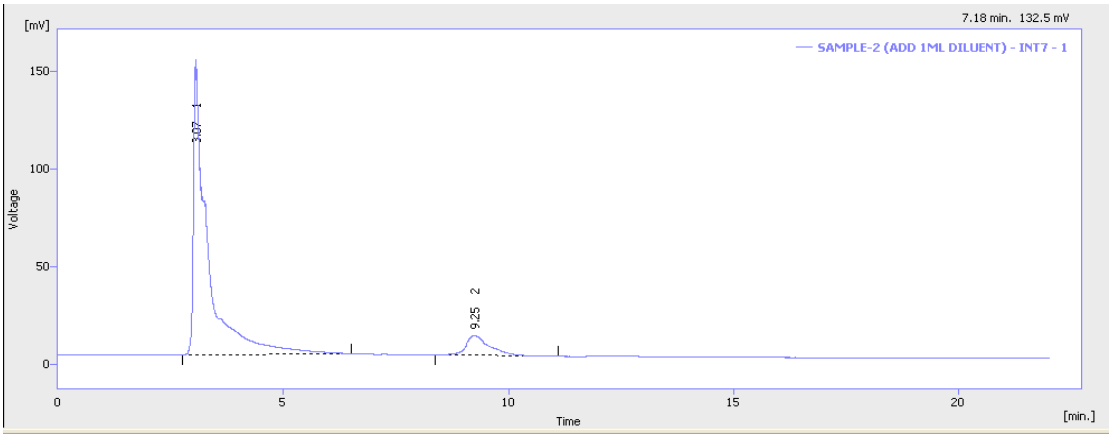

Figure 18: HPLC Chromatogram of green colored pigments.

CONCLUSIONS

This study deals with the pigment production and extraction from pseudomonas aeruginosa at suitable temperature, pH and incubation period. The results of shows the pigments can be produced in laboratory. The results of antibacterial and hemolytic activity were also favorable. The color obtained with agar also pleasant to see. Hence, the pigments seem to have the potential to use as food colorants. HPLC and UV/Visible analysis also carried out for the pigments. However, further research is needed before using these pigments as food colorants in human food.

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