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Identifying the Possibility of Food Grade Pigments from Halophytic Bacteria Isolated from Marine Salterns

[*1]Abbiramakiruthi DK and [2]Dr. N Hemashenpagam

[*1, 2]Department of Microbiology, Hindusthan College of Arts and Science, Coimbatore, Tamil Nadu, India.

Abstract

Microbial pigments have gained significant attention due to their diverse applications in various industries, including food, cosmetics, pharmaceuticals, and textiles. However, the conventional methods used for the biosynthesis of these pigments often rely on nutrient-rich media, which can be expensive and hinder large-scale production. To address this challenge, there is a necessity to develop high throughput and cost-effective approaches for the large-scale production of microbial pigments. This article explores the analysis of the bio pigment production and to assess its bioactive potential of the halophytic bacteria which is isolated from marine salterns by using solvent extraction and purification methods Pseudomonas *aeroginosa*, a halophytic bacteria. Is used as a sample to extract the pigments such as pyocyanin and pyorubrin which was collected from Kovalam salterns, Chennai. The antagonistic effect and hemolytic activity of the pigment have also been carried out. Compared to synthetic pigments, microbial pigments shows better biodegradability and higher compatibility with the environment, and have numerous applications from food to cosmetics.

Keywords: Microbial pigments, pyorubrin, pyocyanin, halophytic bacteria

1. Introduction

Pigments are the chemical substances that absorb the light of visible region. The produced color is because of the chromophore, which captures the sun energy and causes an excitation of electron where the non-absorbed energy is refracted or reflected to be captured by eye. Pigments are compounds with uniqueness of importance to many industries. In the food industry they are used as additives, antioxidants, color intensifiers, etc. Pigments come in a wide selection of colors, some of which are water-soluble.

The benefits of pigment production from microorganisms include easy and fast growth in the cheap culture medium, independence from weather conditions and colors of different shades. Hence, it is one of the promising and emerging fields of research to reveal its potential for various industrial applications. For industry there is a necessity to develop and cost-effective approaches for large scale pigment production. Conventional media have variety of nutrients. Microorganisms vary in their needs to carbon sources according to their nutrient nature; the use of pure carbon sources e.g. (glucose, sucrose, and fructose) is expensive. so the industry try to use contemptible carbon sources especially industrial wastes, variety of plant seed oils etc. Fungal compounds therefore have potential for the direct production of textile dyes or dye intermediates replacing chemical synthesis as they often show mildew growth on textiles, plastics & plastics also have shown anthraquinone derivatives resembling the important class of vat dyes. Some bacteria produce pigments therefore aids in identification of bacteria some pigments are water soluble & others are fat soluble.

The yellow pigment from zeaxanthin from Flavobacterium species can be used as an additive in poultry feed to fortify the yellow color of the skin of birds or to accentuate the color of the yolk of the egg. They can also be used in cosmetic and in food industry. Halophilic bacteria are organisms that thrive under high salt environments such as solar salterns, salt lakes and salt mines which contain large populations of these organisms. The metabolic patterns of halophiles are diverse when compared to their terrestrial counterpart and obligate halophiles

2. Methodology

Sample Collection: Sediment samples were collected from saline environment from Kovalam salterns, Chennai using sterile spatula and aseptically transferred in to sterile polythene bag.

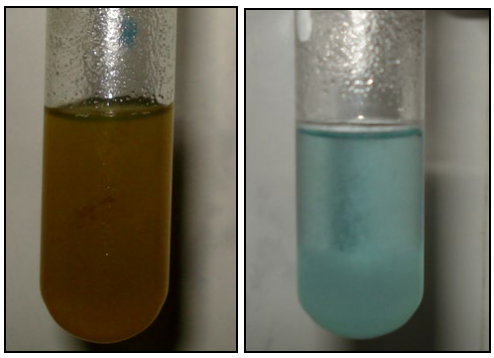
***Corresponding Author:** Abbiramakiruthi DK

Fig 1: Kovalam Salterns, Chennai

Isolation of Halophytic Bacteria: The sediment samples were serially diluted and plated on Zobell Marine agar and incubated at 35oC for 48 h. All the blue green pigmented colonies were transferred to Pseudomonas isolation agar and Cetrimide Agar to ensure the results of blue green pigment production. Isolated strains were purified on Zobell Marine agar and stored in slants with same medium at 4oC until use.

Pigment Production: P. aeruginosa was grown in the medium. 200 ml of the medium was prepared in 500 ml of culture flask and the strain was inoculated as 1% concentration and incubated at 35oC for 72 h.

Extraction of Pigments: Pyocyanin Two volumes of chloroform were added to one volume of cell free culture supernatant and shaken well. The pyocyanin was then extracted from the chloroform into 0.2 N HCl to this deep red acid solution 0.4 M borate-NaOH buffer (pH 10) was added until the colour changed to blue and the blue coloured pyocyanin was again extracted into chloroform. This step was repeated 2 or 3 times, resulting in a clear blue solution of pyocyanin in chloroform and pyocyanin powder was collected by evaporating the chloroform. Pyorubrin After the extraction of pyocyanin in chloroform phase, pyorubrin in the aqueous phase was separated and lyophilized



(a) **(b)**

Fig 2: a) Pyorubrin and **b).** Pyocyanin as food colorants in agar.

Pigment Concentration: Pyocyanin A standard pyocyanin pigment graph was prepared by plotting known concentration of pyocyanin pigment and the OD was measured at 520 nm. Pyocyanin was extracted from the culture broth collected at different time intervals and estimated by measuring the OD at 520 nm and the concentration was calculated from the standard graph. The P. aeruginosa was cultured in mineral salt media. 10 ml culture sample was collected at every 6 hour interval until 72 hours in 30 ml screw cap tubes which was sterilized previously in an autoclave. The pigment pyocyanin was extracted from the culture. OD of the samples were measured at 520 nm and its concentration was estimated by plotting the values in the standard graph. Pyorubrin extracted from the culture broth collected at different time intervals was estimated by measuring the OD at 520nm and the concentration was calculated from the standard graph. A standard pyorubrin pigment graph was prepared by plotting known concentration of the pigments.

Product Formulation: The pigments were extracted and estimated. Various concentrations of the pigment was prepared (5, 10, 15, 20 and 25 mg/ml). 15 ml of different concentrations were taken and 3% agar was added to it. It was then heated to boiling and then cooled to solidification. This is to check whether the pigments give an appealing colour in food materials.

Antibacterial Activity Test (Well Diffusion Method): Antibacterial assay was carried out by well diffusion technique for each pigment along with a control i.e. chloroform for pyocyanin and distilled water for pyorubrin. After incubation for 24 h at 37oC, the inhibition zone was measured and recorded. Antibacterial activity of P. aeruginosa was determined against bacterial pathogens viz. Salmonella paratyphi, Escherichia coli, Citrobacter sp. and Klebsiella pneumonia.

Antibacterial Activity Test: Nutrient broth medium was prepared and sterilized and the above human pathogenic strains were inoculated and incubated at 37oC for 24 h. Sterilized Muller Hinton agar plates were prepared and the pathogens were swabbed on the surface of the agar and the P. aeruginosa strain was inoculated by streaking on the surface agar. The plates were incubated for 24-48 h at 37oC and observed for antibacterial activity.

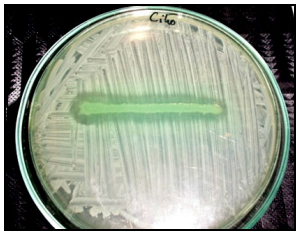


Fig 3(a): P. aeroginosa culture

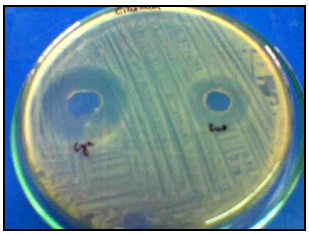
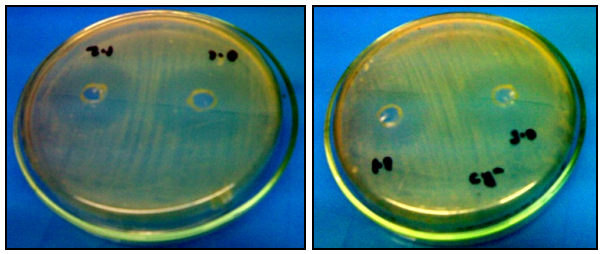
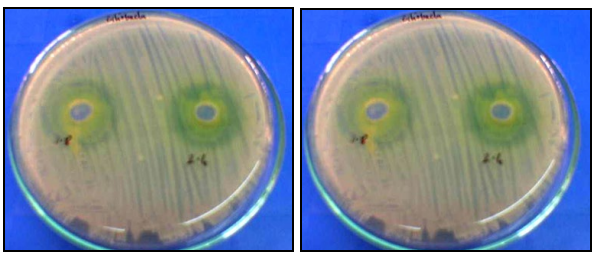


Fig 3(b): Crude pigments



(c) **(d)**

Fig 3(c & d): Different concentration of pyocyanin,



(e) **(f)**

Fig 3(e & f): Differs concentration of pyorubrin

Preparation of Pigment Extracts (Drying): Pyocyanin the pyocyanin pigment extracted with chloroform was air dried. To the dried extract 10 ml of chloroform was added and different concentration (5, 10, 15 20 and 25 mg/ml) of the pigment was prepared with chloroform. Pyorubrin From the lyophilized sample different concentration (5, 10, 15 20 and 25 mg/ml) of the pigment was prepared with distilled water.

Hemolytic Activity of Phenazine Pigments: Preparation of erythrocyte suspension Fresh sheep blood was collected from a nearby slaughter house and EDTA (2.7 g in 100 ml distilled water) was added as anticoagulant at the rate of 5% of the volume of blood. The blood was centrifuged at 5000 rpm for 7 minutes at 4oC along with normal saline and the supernatant was discarded. 1 ml of the packed RBC thus obtained was resuspended in normal saline to obtain a 1% RBC suspension. The same procedure was adopted for chicken blood also.

Hemolytic Assay: The assay was carried out in "V" shaped Laxbro microtitre plates. The lyophilized pyorubrin pigment and the air dried pyocyanin pigment were assayed. Various concentration of the pigment was prepared. One row of well was used for only one concentration. Initially 100 µl of normal saline was added to each well. Then 100 µl of the lowest concentration of the pyocyanin pigment was added to the first well and this process was repeated up to the last well from which 100 µl was discarded. Then 100 µl of the prepared erythrocyte suspension was added to each well. A negative control was kept by mixing 100 µl of normal saline and 100 µl of 1% RBC suspension. Formation of a fine "Button cell" with regular margin indicates the negative reaction. A uniform red coloured suspension of the lysed RBC indicates the positive result. The plates were incubated for 3 hours at room temperature and the results were observed. Hemolytic activity was expressed as hemolytic unit (HU).

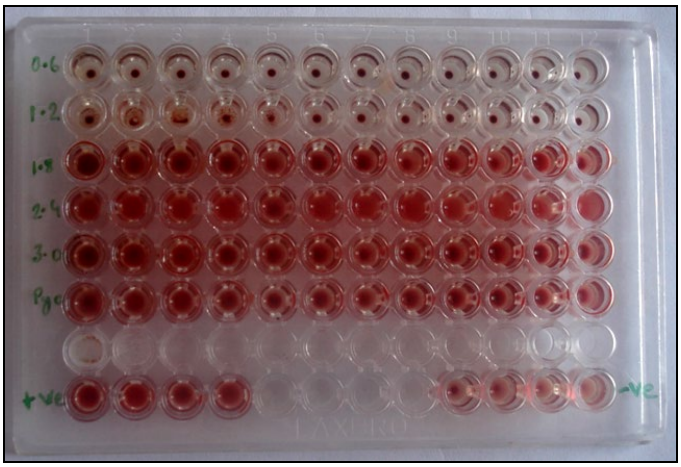


Fig 4: Hemolytic activity of pigments against chicken blood.

3. Results and Discussion

Out of the 31 different colonies isolated in Zobell marine agar medium, only two strains showed growth in Pseudomonas isolation medium and also produced blue green diffusible pigment. These two isolates were subjected to morphological, cultural, biochemical and physiological characterization. Based on the results the isolates were identified as Pseudomonas aeruginosa. Out of the two strains producing pigments, one strain was chosen for the present study as it showed comparatively intense pigment production.

Pigment Production and Characterization: Pigment production in mineral salt medium revealed that the appearance of pigment starts at 6th h. The pyocyanin and pyorubrin was estimated at six hour interval. Pigments showed steady increase in concentration up to 60 h of incubation and decreased afterwards. The initial pyocyanin production was comparatively more than pyorubrin. The initial pyocyanin pigment concentration at 6 h was 3.50 mg/ml whereas the initial concentration of pyorubrin was 2.50 mg/ml. The rate of increase in pigment concentration was nearly equal for both the pigments. There is a correlation observed between the biomass and pigment production up to 66 h after which decrease in biomass was observed.

Pigments as Food Colourants: In pyocyanin, all the concentration showed good, pleasant colouration. The maximum intensity was observed in 25 mg/ml. and the least colouration was observed in 5 mg/ml. with pyocyanin. Even in lower concentration the colour obtained which was pleasant to see. Likewise pyorubrin showed a chocolate brown colour and other things holds good with that of pyocyanin.

Antibacterial Activity of Pyocyanin and Pyorubrin: The pigment extracts from P. aeruginosa showed distinct antibacterial activity against Citrobacter sp. The pigments did not show antibacterial activity against other pathogens tested. The antibacterial activity was found in all the concentrations (5, 10, 15, 20, 25 mg/ml) of pyocyanin and pyorubrin pigments. The lower concentrations of both the pigments showed lower inhibitory zone when compared to the higher concentration. The maximum inhibition zone was found with pyocyanin pigment (1.5cm) and the minimum inhibition zone was observed with pyorubrin pigment (1 cm).

4. Conclusion

The selected strain was isolated from sediment sample of Kovalam salterns, Chennai. The present study includes the isolation and identification of a P. aeruginosa strain from salt environment, estimation of the phenazine pigments i.e. pyocyanin and pyorubrin produced from it followed by checking for antibacterial effect and hemolytic activity.

The purpose behind the work is to check the potential of the pigments of P. aeruginosa to use as food colourants. Estimation of pigment production and growth were carried out in mineral salts medium and the strain was cultured for 72 h. The pigments and biomass showed steady increase in concentration up to 60 and 66 h respectively.

When the pigments were checked against bacterial pathogens viz. Salmonella paratyphi, Escherichia coli, Citrobacter sp. and Klebsiella pneumonia, antibacterial effect was found only against Citrobacter sp. Though Citrobacter sp. is not a potent human pathogen, it is recorded as an opportunistic pathogen resulting in diseases like urinary tract infections, wound infections and sometimes pneumonia in humans especially in immunocompromised persons.

The maximum activity was noted with pyocyanin. Further study on antibacterial activity of pigments against more number of human pathogens is needed to prove the antibacterial property of the pigments. This experiment was done to check along with colouring the food whether the pigments can also act as a preservative and as a probiotic. Both the pigments showed hemolytic activity at 15, 20 25 mg/ml concentrations against chick blood, but no activity was found at 5 and 10 mg/ml concentration with chicken blood.

However in sheep blood no hemolytic activity was found. Thus the results of the present study clearly indicated that the phenazine pigments, pyocyanin and pyorubrin of P. aeruginosa can be produced in large quantities by the strain used and no special environment is needed for the production. The results of antibacterial and hemolytic activity were also favourable. The colour obtained with agar also pleasant to

see. Hence the pigments seem to have the potential to use as food colourants.

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