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Phenazine Pigments from *Pseudomonas aeruginosa* and Their Application as Antibacterial Agent and Food Colourants

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Abstract: This study deals with isolation and identification of *Pseudomonas aeruginosa* from marine environment for phenazine pigment production. Pigment production results revealed that, the strain could able to produce two different pigments namely, pyocyanin and pyorubrin. Maximum biomass was observed at 66 h of incubation, but the pigment production seems to be continuous throughout the culture period (72 h). Antibacterial activity of the pigments was evaluated against pathogenic bacteria, maximum growth inhibitory activity was observed with pyocyanin and pyorubrin at 20 μL concentration (1.7 and 1.3 cm, respectively) against *Citrobacter* sp. Hemolytic activity of the pigments inferred that, hemolysis was observed with both pigments at 15, 20 and 25 μL concentration and no hemolysis was found at 5, 10 and 15 μL. The pigments were evaluated for their potential as food colourants with agar. Pleasant colouration was observed with pyocyanin and pyorubrin at 25 mg mL^{-1} concentration.

Key words: Pyocyanin, pyorubrin, phenazine, pigment, *Pseudomonas aeruginosa*

INTRODUCTION

Nature is rich in colours (minerals, plants etc.) and pigment producing microorganisms (fungi, yeasts and bacteria). Microbial pigments such as carotenoids, melanins, flavins and quinines are used in many food industries (Dufosse, 2006). The ability to synthesis pigments varies with the organism and its environment. Fluorescent Pseudomonads are ubiquitous bacteria that are common inhabitants of the water, soil, plants and animals and they are the most studied group.

Pseudomonas aeruginosa produces variety of extracellular pigments, of which phenazines comprise a significant portion. Phenazine compounds produced by fluorescent *Pseudomonas* species are biologically active metabolites that function in microbial competitiveness (Mazzola *et al*., 1992). Strains of *P. aeruginosa* produce variety of redox-active phenazine compounds, including pyocyanin, phenazine-1-carboxylic acid and phenazine-1-carboxamide (Budzikiewicz, 1993). Pyocyanin is the main phenazine pigment associated with this particular organism. About 90 to 95% of *P. aeruginosa* strains produce pyocyanin (Smirnov and Kiprianova, 1990). Defined conditions for the reliable production of phenazines have greatly aided by biosynthetic studies (MacDonald, 1967). The simultaneous accumulation of more than one phenazine by *P. aeruginosa* had been reported (Korth *et al*., 1978) and culture conditions which differentially affect the fractional composition of phenazine pigments had also been described (Kanner *et al*., 1978). Pyocyanin inhibits Nitrous Oxide (NO) synthesis and blocks the relaxation of rabbit aortic smooth muscle induced by NO and organic nitrates. Pyocyanin has a variety of pharmacological effects on eukaryotic and prokaryotic cells (Vukomanovic *et al*., 1997).

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P. aeruginosa secretes proteins which are toxic to a wide range of organisms and it also secretes copious amount of phenazine pigments (Chang and Blackwood, 1969). Phosphate depletion within this microbe has been suggested to be the initiator of phenazine biosynthesis. Pyocyanin interferes with the regulation of ion transport, ciliary beat frequency and mucus secretion in airway epithelial cells by altering the cytosolic concentration of calcium (Denning *et al*., 2001). Pyocyanin is known to suppress wheat blotch caused by *Septoria tritici* (Flaishman *et al*., 1990). Phenazines compounds produced in the rhizosphere of plants contribute to the biological control activity of *P. aeruginosa* against *Fusarium* wilt of chickpea and *Pythium* damping of bean (Anjaiah *et al*., 1998). Due to the abundant occurrence and considering the biotechnological application of *P. aeruginosa* the present study was under taken with the following objectives: isolation and identification of *Pseudomonas aeruginosa* from marine environment, estimation of pyocyanin and pyorubrin pigment production. Application of pigments as food colourants and antibacterial effect of the pigments against pathogenic bacteria.

MATERIALS AND METHODS

Isolation and Identification of *P. aeruginosa*

Sediment samples were collected in the mangrove environment from Vellar estuary (Lat. 11° 29'N; Long. 79° 46'E) using sterile spatula and aseptically transferred in to sterile polythene bag and further analysis was carried out in Centre of Advanced Study in Marine Biology, Parangipettai, Tamil Nadu, India during January to March 2007. The sediment samples were serially diluted and plated on Zobell Marine agar and incubated at 35°C for 48 h. All the blue green pigmented colonies were purified on Zobell Marine agar and stored in slants with same medium at 4°C until use. Identification of *P. aeruginosa* was done based on morphological, cultural, biochemical and physiological characteristics as suggested by Cappuccino and Sherman (1999) and Schaad *et al*. (2001) and the results were crosschecked with Bergey's Manual of Determinative Bacteriology (Buchanan *et al*., 1974).

Pigment Production

P. aeruginosa was grown in the medium described by Frank and DeMoss (1959). The mineral salt medium contains (g L^{-1} until other wise specified) DL-Alanine-10, Glycerol-20 mL, K_2HPO_4-0.139, $MgCl_2.6H_2O$-4.06, Na_2SO_4-14.2, Ferric Citrate-0.1, 50% seawater-1000 mL and pH 7±0.02. Sterilized medium was inoculated with 1.0% of 18 h culture and incubated at 35°C for 72 h.

Extraction, Estimation of Pyocyanin and Pyorubrin Pigments and Biomass

Two volumes of chloroform was 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 (Frank and DeMoss, 1959). After the extraction of pyocyanin in chloroform phase, pyorubrin in the aqueous phase was separated and lyophilized (Palumbo, 1972).

A standard pyocyanin and pyorubrin pigment graph was prepared with known concentrations of pigments. Concentration of pigments in the culture broth was estimated by measuring the OD at 520 nm and the obtained OD values were compared with the standard graph and the concentration of the pigments were expressed as mg mL^{-1}.

Broth culture samples collected at different intervals were centrifuged at 10,000 rpm for 15 min to obtain the cell pellet. The cell pellet was dried in hot air oven at 80°C for a period of 24 h and the biomass was quoted in terms of mg mL^{-1} (dry weight).

Pigments as Food Colourants

Various concentrations of pyocyanin and pyorubrin were prepared (5, 10, 15, 20 and 25 mg mL^{-1}) and 3% of agar was added to the pigment solution and heated to boiling and then cooled for solidification. This test was made to ensure the reliability of the pigments and their colouring ability at boiling temperature in food materials.

Antibacterial Assay

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. Wells with 0.5 cm diameter were made on sterile Muller Hinton agar plates. Pathogenic bacteria namely *Salomonella paratyphi*, *Escherichia coli*, *Citrobacter* sp. and *Klebsiella pneumonea* were swabbed on the surface of the agar and different concentration of pyocyanin/pyorubrin pigments (5, 10, 15, 20 and 25 μL) were added in to the wells. Plates were incubated at 37°C for 24 h. Antibacterial activity of the pigments was determined by measuring the growth inhibition around the well.

Hemolytic Activity of Phenazine Pigments

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 min at 4°C 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.

The assay was carried out in Laxbro microtitre plates. The lyophilized pyorubrin and the air dried pyocyanin pigments 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 h at room temperature and the hemolysis was observed.

RESULTS

Isolation and Identification of *P. aeruginosa*

Out of 31 different colonies isolated in Zobell marine agar medium, only two strains showed luxuriant 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* (Table 1). 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 6 h in the culture medium. The pyocyanin and pyorubrin was estimated at 6 h intervals. Pigments showed steady increase in concentration through out the incubation period. The initial pyocyanin production was comparatively more than pyorubrin. The initial pyocyanin pigment concentration at

Table 1: Physiological and biochemical characteristics of *P. aeruginosa*

Tests	Results
Colony	Circular, raised, smooth margin
Pigment on nutrient agar	Diffusible green pigment turning blue green at 48 h
Gram reaction	Negative
Motility	Motile
Cell shape and arrangement	Small rods, mostly single
Endospore	Not formed
Growth at 48°C	+
Relation to oxygen	Aerobic
Citrate utilization	+
Catalase	+
Oxidase	+
Methyl red	-
Vogus proskuaer	-
Indole production	-
H_2S production	+
Starch hydrolysis	+
Lipid hydrolysis	+
Gelatin liquefaction	+

+: Positive, -: Negative

Table 2: Pyocyanin and pyorubrin production by *P. aeruginosa*

Time (h)	Biomass	Pyocyanin (mg mL^{-1})	Pyorubrin
0	0	0.00	0.00
6	10	3.50	2.50
12	15	4.00	3.00
18	20	4.00	3.25
24	30	4.00	4.00
30	36	4.00	4.25
36	41	4.75	4.50
42	46	4.75	4.50
48	49	4.75	4.50
54	50	4.75	4.75
60	52	5.25	4.75
66	60	5.25	5.00
72	57	5.25	5.50

6 h was 3.50 mg mL^{-1} whereas the initial concentration of pyorubrin was 2.50 mg mL^{-1}. There was a correlation observed between the biomass and pigment production up to 66 h after which decrease in biomass concentration was observed (Table 2).

Pigments as Food Colourants

In pyocyanin, all the concentration showed good, pleasant colouration. The maximum intensity was observed in 25 mg mL^{-1} (Fig. 1) and the least colouration was observed in 5 mg mL^{-1} with pyocyanin. Even in lower concentration the colour obtained with the pigments was pleasant to see. Like wise pyorubrin showed a chocolate brown colour and other things holds good with that of pyocyanin.

Antibacterial Activity of Pyocyanin and Pyorubrin

Pigment extracts from *P. aeruginosa* showed distinct antibacterial activity against *Citrobacter* sp. Antibacterial activity was found in all the concentrations (5, 10, 15, 20 and 25 mg mL^{-1}) of pyocyanin and pyorubrin pigments. 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 (1.7 cm) and the minimum inhibition zone was observed with pyorubrin pigment (1 cm) (Table 3).

Table 3: Antibacterial activity of pyocyanin and pyorubrin against *Citrobacter* sp.

Concentration of pyocyanin and pyorubrin (mg mL^{-1})	Diameter of inhibitory zone (cm)	
	Pyocyanin	Pyorubrin
5	1.4	1.0
10	1.5	1.0
15	1.5	1.2
20	1.6	1.2
25	1.7	1.3

Table 4: Hemolytic activity of pyocyanin and pyorubrin pigments

Concentration of pigments (mg mL^{-1})	Results	
	Pyocyanin	Pyorubrin
5	-	-
10	-	-
15	+	+
20	+	+
25	+	+

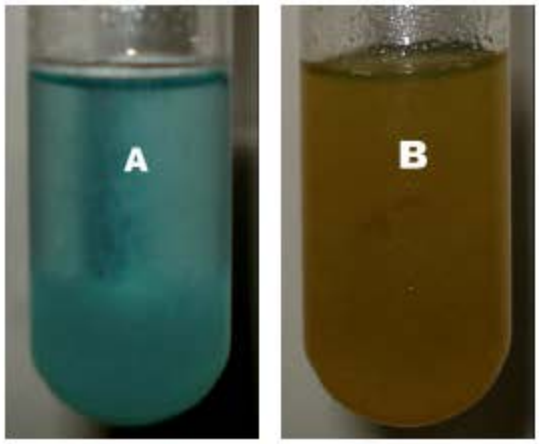

Fig. 1: Pyocyanin (A) and pyorubrin (B) mixed with agar

Hemolytic Assay

Pyocyanin and pyorubrin pigments were found to be hemolytic positive for the concentration of 15, 20 and 25 mg mL^{-1} (Table 4) in chicken blood. There was no hemolytic activity at 5 and 10 mg mL^{-1} concentration of both the pigments. With sheep blood no hemolytic was observed even in higher concentration i.e., 25 mg mL^{-1}.

DISCUSSION

Isolation of *P. aeruginosa* was carried out in Zobell marine agar. Out of 32 colonies, only 2 colonies (6.25%) showed intense blue green pigmentation and identified as *P. aeruginosa*. The strain which showed intensive pigment production was used for further study. The strain produced two pigments, one with blue colouration and another with greenish brown colour named respectively as pyocyanin and pyorubrin.

Regarding pigment production, the pyocyanin and pyorubrin pigments showed a steady increase in concentration throughout the culture period (72 h) and these results were comparable with that of Graham *et al*. (1979). In the present study, the stability of pyocyanin pigment concentration after 60 h maybe due to the range of phenazine compounds produced in the growth phase or in response

to environmental signals. The biomass showed it's maximum at 66 h and attained declined phase at 72 h, the close association between growth and pigment production from 6 to 66 h effectively showed the utilization of substrate for growth as well as for pigment production.

Both the pigments of *P. aeruginosa* showed distinct antibacterial effect against *Citrobacter* sp. However greater effect was found with pyocyanin pigment. Production of antibiotics by *P. aeruginosa* has been recognized as a major factor in controlling many pathogens. The phenazine compounds are phenolic in nature, which includes pyocyanin and pyorubrin pigments, exhibit broad spectrum antibiotics. Phenazines are synthesized from chorismic acid, possibly via anthranilate, which is also an intermediate in the tryptophan biosynthesis pathway (Essar *et al*., 1990). Anthranilate is synthesized from chorismate by two distinct anthranilate synthases in *P. aeruginosa*. Phenazine B, whose deduced amino acid sequence is similar to those of anthranilate synthases, required for phenazine antibiotic production in *P. aureofaciens* (Pierson and Pierson, 1995). *P. aeruginosa* PNA1, when grown in culture, produced phenazine antibiotics phenazine-1-carboxylic acid and oxychloraphine and inhibited the mycelial growth of *F. oxysporum*, *P. splendens* and other phytopathogenic fungi (Anjaiah *et al*., 1998). *Citrobacter* a member of the family Enterobacteriace, may cause urinary tract infections, wound infections and some times pneumonia in humans especially in immunocompromised persons (Hodges *et al*., 1978). In urinary infection *Pseudomonas* sp. and *Citrobacter* sp. co-exit in this regard the present result may be valuable.

The idea of checking the pigment's antibacterial activity against water and food borne pathogens was whether it has an additional protective effect when used as a food colourant. Results obtained in the present study were not encouraging, because the pigments showed antibacterial activity only against *Citrobacter* sp. However this cannot deter the pigments in using them as colourants.

Hemolytic assay inferred that both the pigments did not show any hemolytic activity at lower concentrations (5 and 10 mg mL^{-1}) and at 15, 20 and 25 mg mL^{-1} concentrations hemolysis was found in chick blood. Though it produced hemolysis in chick blood, no hemolysis was observed in sheep blood. However feeding experiments with other animal models are needed before using them in food intended for human consumption or to incorporate them in animal feeds.

Pyocyanin pigment from *P. aeruginosa* produced good colouration when mixed with agar. It showed a light blue colour which did not deterioated on boiling. The pigment did not make the agar to loose its property. The pigment seems to have a binding property with agar. Pyorubrin also had all these properties except that it gave chocolate brown colour on boiling. Many microbial pigments have already been commercialized: Astaxanthin from *Xanthophyllomyces dendrorhous*, pink red from *Penicillium oxalicum*, riboflavin from *Asbhya gossypii*. Like wise *P. aeruginosa* pigments also has the potential to use as colourant in beverages, cakes, confectionaries, puddings etc. the pigments may also be used to decorate and display the food items also.

Thus the results of the present study clearly indicated that the phenazine pigments, pyocyanin and pyorubrin of *P. aeruginosa* can be produced in laboratory. 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. However, further research is needed before using these pigments as food colourants in human food.

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