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Research Article

MICROBICIDAL RESPONSE OF PYOCYANIN PRODUCED BY *P. aeruginosa* TOWARD CLINICAL ISOLATES OF FUNGI

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ABSTRACT

Objective: Pseudomonads are well known for their degradative abilities and play an important role in the environmental cleanup and they are opportunistic pathogens. The characteristic feature of *Pseudomonas aeruginosa* is the production of soluble pigments like pyocyanin, fluoresceins and exo-polysaccharides, a secondary metabolite that is produced in both solid and liquid culture media. The study aims at production and characterization of pigment pyocyanin and to evaluate its antimicrobial potential toward clinical isolates of fungi.

Methods: Production of pyocyanin using Pseudomonas broth was validated. Extraction of the pigment was done by chloroform extraction method. Uv-visible absorption spectrum and Gas Chromatography were used to characterize the pigment and its components. Structural elucidation of pyocyanin was done using Nuclear Magnetic Resonance (NMR). Further, the antimicrobial activity of the pigment was evaluated using Cross streak method.

Results: Pigment production was achieved after 24 h of incubation with a color change to bluish green. UV-visible spectra revealed a maximum absorption at 278nm characteristic of pyocyanin. The molecular weight of the compound was determined as 210.23kDa with a retention time of 11.94 min. NMR study revealed the presence of methyl group linked to condensed nitrogen aromatic ring. The antifungal activity of the pigment was found maximum toward *Candida* sp. and *Cryptococcus neoformans*.

Conclusion: The properties of the pigment make it an important bioactive compound which has the ability to arrest the electron transport chain of fungi and exhibit antifungal activity towards *Candida* sp., and *Cryptococcus neoformans.*

Keywords: Pyocyanin, GC-MS, NMR, Antifungal activity.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen of human [1,2] belonging to the family pseudomonadaceae. It is widespread in the environment wherein majority of which are responsible for nosocomial infections. In addition, they are also found associated with chronic infections in cystic fibrosis patients and wound infections especially of burns [3]. The bio-film formation on various antiseptics and disinfectants and the mechanism involving drug resistance toward various antibiotics has been one of the strategies in the emergence and the release of the resistant strain in the environment [4]. However, Pseudomonads are known to produce pigments resolved to survive under oxidative stress imposed by environmental hazards and exhibits antagonism.

The characteristic feature of *Pseudomonas aeruginosa* is the production of soluble pyocyanin pigment, a water soluble blue green phenazine compound produced in large quantities. Pyocyanin has antibiotic activity against bacteria, fungi and protozoa. *P. aeruginosa* was found to produce various phenazine pigment identified as pyocyanine, phenazine-1-carboxylic acid, 1-hydroxy phenazine and phenazine-1-carboxamide [5]. The present study deals with biosynthesis, purification and characterization of pyocyanin pigments produced by *P. aeruginosa.* The purified pigment was used as bioactive compound to study the *in vitro* antagonistic activity of pyocyanin pigment against various molds and yeast.

MATERIALS AND METHODS

Bacterial strain

P. aeruginosa, a clinical isolate used in this experiment was provided by Sharp clinical laboratory, Chennai. The culture was maintained in nutrient agar slants at 4ºC.

Production, extraction and characterization of pyocyanin

P. aeruginosa was inoculated in Pseudomonas broth to optimize the maximum production of pyocyanin. They were incubated at 37ºC on a rotary shaker for 24 h and observed for color change. The broth culture prior to color change was centrifuged at 10,000rpm for 5 min at 4ºC. The pigment was extracted using chloroform (1:2) and the aqueous phase discarded. To the solvent phase 0.2 N HCl was added and the color change observed.

The pigment was further characterized using UV-visible spectrophotometer (UV T-1800) by sampling 2ml aliquot of the pigment and the absorption maxima observed.

The GC-MS analysis was done with standard specification by dissolving 100mg of pyocyanin with 1ml chloroform. The liquid sample of 1ul was injected into column of GC-MS model (Joel GC-Mate II Mass spectrometer) HP5 silica column as stationary phase and helium as a carrier gas with the flow rate of 25ml / min. The maximum peak representing mass to charge ratio characteristics were compared with those in the mass spectrum library of the corresponding organic compounds. The structural elucidation of the pigment was determined using Nuclear Magnetic Resonance by dissolving the compound in $CdCl_3$.

Demonstration of Antifungal Activity

The following fungal cultures were used for the demonstration of antifungal activity namely, *Candida albicans, Candida krusei, Candida glabrata, Candida tropicalis* and *Cryptococcus neoformans*.

P. aeruginosa inoculum was streaked diametrically across Sabouraud's dextrose agar plates and was then incubated at 37ºC for 24 h. The growth on the surface of the agar plate was then removed with a sterile cotton swab. Each plate was then placed upside down with chloroform soaked filter paper disc on the lid and was left for 30 min so that the traces of the cells were killed. The filter paper was removed from the plate and the traces of chloroform were eliminated on exposure to flowing air for a few minutes in laminar air flow. A fresh 24 h plate culture of the test strains was used to prepare the inoculum. This yeast suspension was streaked onto the chloroform treated SDA plates at right angle to the original inoculum. Plates were then incubated for 24 h at 30ºC for the demonstration of inhibition of fungal growth.

RESULTS

Pigment production was accomplished after 24 h of incubation. Soluble pigment namely pyocyanin production were indicated by

change in color to bluish green. However, the change in color of the pigment to deep pink was observed upon addition of chloroform and 0.2 N HCl. This confirmed the pigment produced was pyocyanin. UV-vis analysis result revealed a maximum absorbance at 278 nm, indicative of pyocyanin compound (Figure 1 & 2).

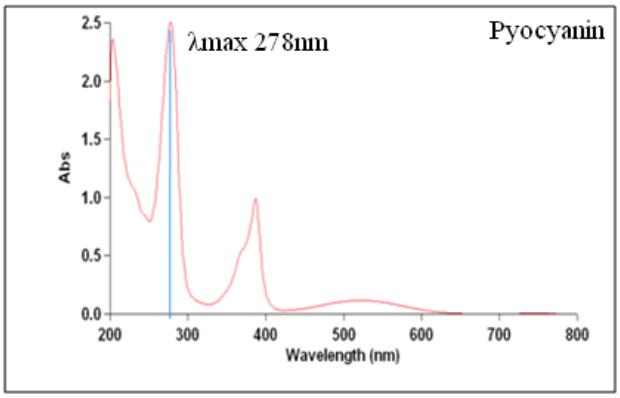

Fig. 1: UV absorption spectra of *P. aeruginosa* showing λ_{max} at 278nm

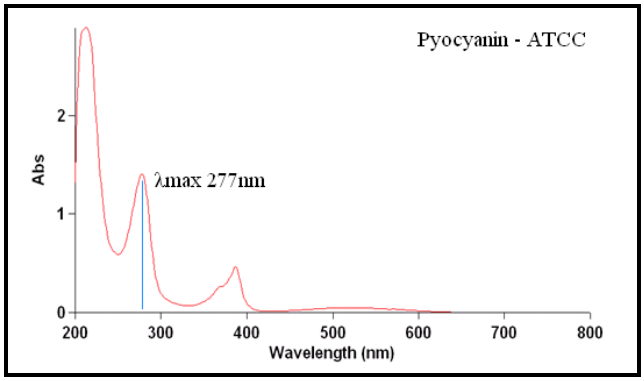

Fig. 2: UV absorption spectra of *P. aeruginosa* ATCC showing λ_{max} at 277nm

GC-MS analysis

Pyocyanin compound which was produced by *P. aeruginosa* was subjected to GC-MS which has the intense molecular ion at m/z 210.23 defining the molecular weight as 210 kDa. The mass spectrum showed intense ions at m/z 210 and other ions 140, 125, 168, 194, 181, 156, 108, 92 (Figure 3 & 4).

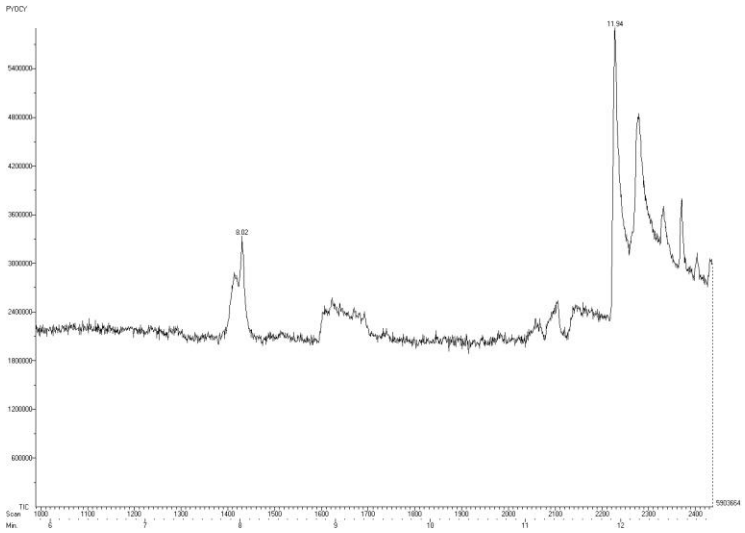

Fig. 3: Gas Chromatogram pattern of pyocyanin pigment

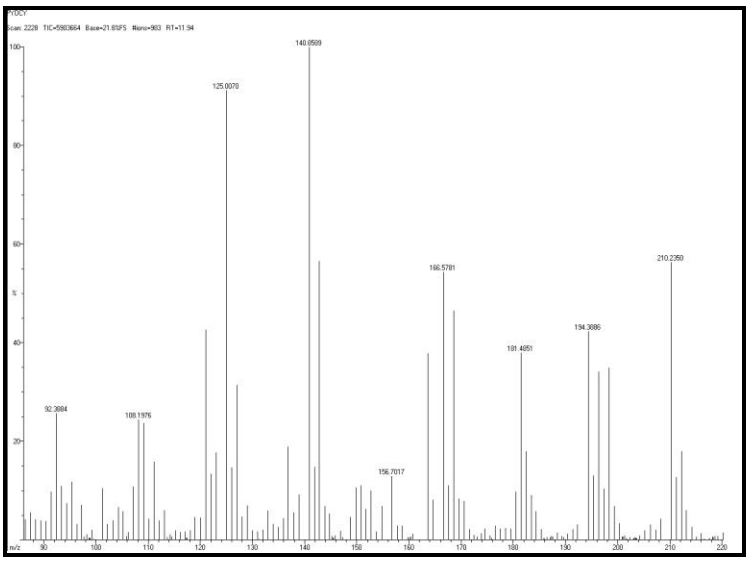

Fig. 4: Mass spectrometry analysis of Pyocyanin pigment

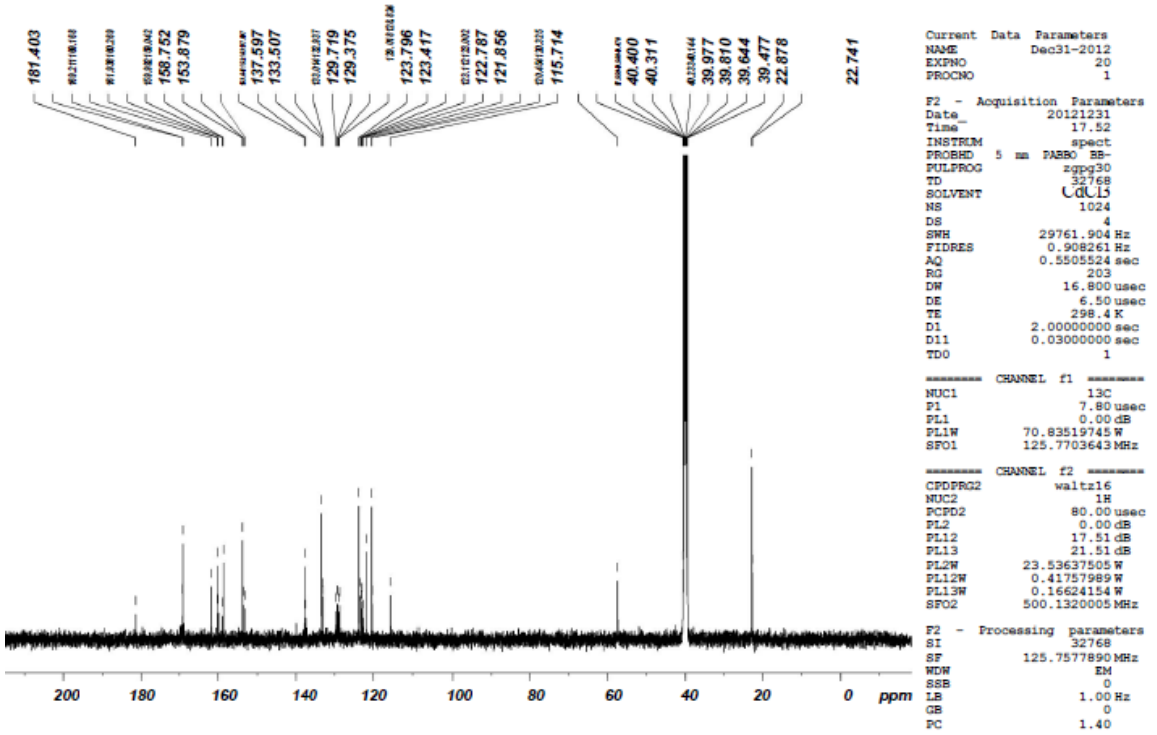

Fig. 5: ^{13}C NMR analysis of Pyocyanin pigment

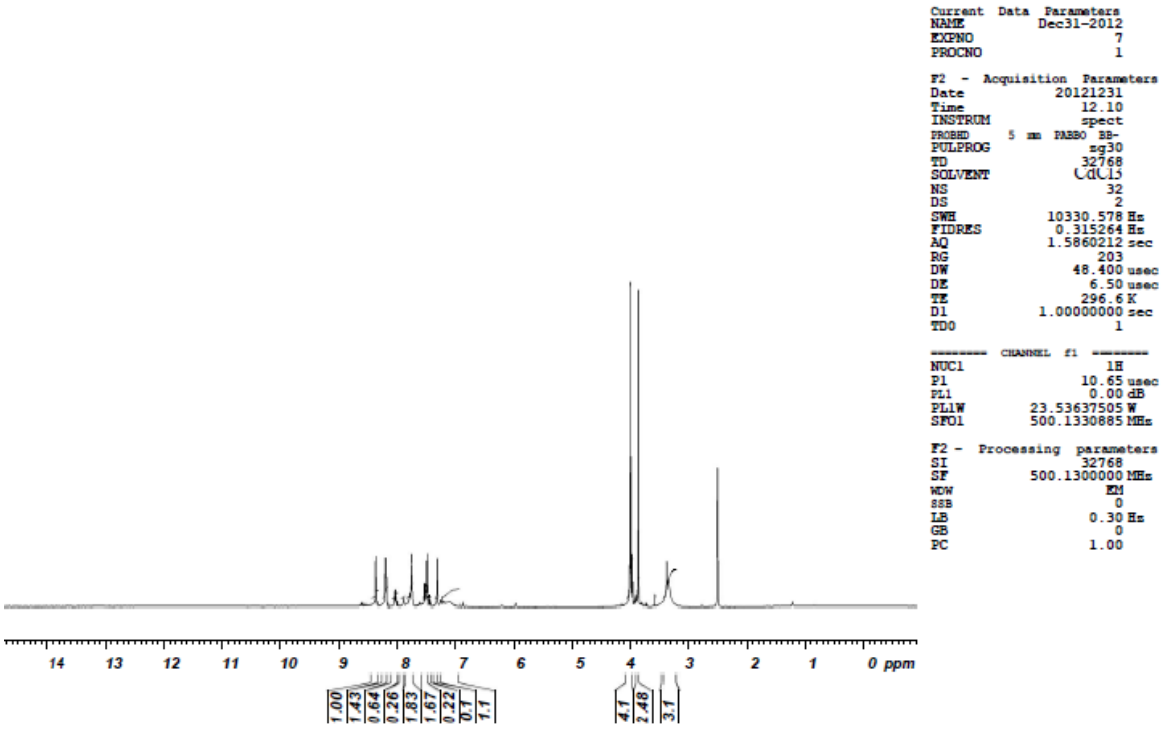

Fig. 6 ^{1}H NMR analysis of Pyocyanin pigment

NMR study

The isolated compound was subjected to both ^{13}C and ^{1}H NMR analysis using $CdCl_3$ as solvent. In ^{1}H NMR (Figure 5), the peak at δ 3.3 to 4.3 represents the presence of Methyl group linked to aromatic nitrogen atom. The peak at δ 7.3 to 8.3ppm indicates the presence of condensed nitrogen aromatic ring. Similarly in ^{13}C NMR, the peak at 120 to 137ppm confirmed the presence of nitrogen aromatic ring as shown in Figure 6.

Antifungal Activity

The pigment produced by *P. aeruginosa* was subjected to antifungal activity against *Candida sp.* and *Cryptococcus neoformans*. On the whole it showed a good antifungal activity (Table 1).

DISCUSSION

The antifungal efficacy of the pigment produced by *P. aeruginosa* was carried out using *in vitro* inhibition assay (cross streak method)

against *Candida species* and *Cryptococcus neoformans.* Enhanced pigment production was observed in Pseudomonas broth indicating the maximum diffusion and soluble nature of the pigment in broth culture. Despite other pigments of Pseudomonads, pyocyanin was considered to be the major antifungal agent with 1-hydroxy-phenazine possessing inhibitory effect. The most widely used criteria for distinguishing *P. aeruginosa* from closely related organisms is by the production of pyocyanin pigment. The change in color of the pigment to deep pink observed upon addition of chloroform and 0.2N HCl confirmed the presence of pyocyanin [6]. The absorbance of this solution was found maximum at 278nm. This peak indicates the presence of pyocyanin compound as compared with the ATCC strain with absorbance maxima at 277nm. GC-MS analysis was done to see the purity of the pyocyanin compound and the result revealed the molecular weight of about 210 kDa [7] and the retention time was found using GC, the eluted peak had a RT of about 11.94 min. This peak was the major peak and a minor peak was also seen this was not taken in our work since the peak was found in negligible amount. The molecular weight of the pyocyanin compound was about 210.23 kDa [8]. Proton NMR and ^{13}C NMR peak clearly indicates the presence of methyl groups linked to the aromatic nitrogen atom [9].

The pigment produced by *P. aeruginosa* was subjected to antifungal activity against *Candida sp.* and *Cryptococcus neoformans*. On the whole it showed a good antifungal activity (Table 1).

Table 1: Antifungal activity of Pyocyanin

S. No.	Fungal Strain	*Pseudomonas aeruginosa*
1.	*C. albicans*	+
2.	*C. krusei*	-
3.	*C. tropicalis*	+
4.	*C. glabrata*	+
5.	*C. neoformans*	+

+ Antifungal activity, – No antifungal activity

Pyocyanin was produced, purified and the nature of its antifungal activity was determined toward yeasts. The apocyanogenic pseudomonas tested was found resistant to the pigment, suggesting the resistance characteristic of the genus. *P. aeruginosa*, the producer organism, was essentially unaffected by high concentration of pyocyanin. Facultative anaerobes were twofold or manifold resistant to the action of the pigment under fermentative conditions. However, the inhibitory effect does not require oxygen since denitrifying bacteria were susceptible during anaerobic respiration than during aerobic respiration.

Previous study [10] reported that *P. aeruginosa* suppressed the growth of *Candida albicans in vitro*. Further *in vitro* susceptibility studies revealed significant inhibition by co-strains of *P. aeruginosa* and fungi known to infect human, these were *C. krusei, C. keyfr, C. guillermondii, C. tropicalis, C. albicans, C. glabrata, C. lusitaniae, C. parapsilosis, C. pseudotropicalis, Saccharomyces cerevisiae* and *Aspergillus fumigatus*. *Pseudomonas spp.* produces a variety of metabolites of which some exhibit antimicrobial activity of which antimicrobial substance pyrrolnitrin has been known to possess antifungal activity [10].

In the present study pigment produced by *P. aeruginosa* possess antifungal activity against *Candida albicans, Candida krusei, Candida glabrata, Candida tropicalis, Cryptococcus neoformans* [11,12]. *Pseudomonas spp.* produces a variety of metabolites of which some exhibit antibiotic activity [13] of which antimicrobial substance pyrrolnitrin has been known to possess this antifungal activity.

CONCLUSION

The purified pigment has bioactive properties and is especially active against fungal pathogens. The clinical strain *P. aeruginosa* could be used to produce the pigment in large quantities and simple purification method makes the culture a promising one in the field of pharmaceutical application. Further strain improvement might be performed to increase the pigment production and apply it as a fungicidal in horticulture or as a biopesticide.

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