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Molecular Characterization and Antifungal Activity of Pyocyanin (Phz A) Gene

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***P. aeruginosa*, an opportunistic pathogen produces antimicrobial substance in the form of a diffusible pigment, pyocyanin. The pyocyanin pigment was extracted using solvent extraction and further characterization studies were performed. The genotypic confirmation of biosynthetic pyocyanin phz A gene was amplified by PCR using suitable primers. The amplified gene corresponded to 217bp sequence. Further, the antifungal activity of the pigment pyocyanin was assessed using various fungal (yeasts) cultures. The antifungal activity of the pigment was found to contain an inhibitory substance, pyrrolnitrin that helps to arrest the electron transport chain of the tested cultures. This pigment can be tailored for an enhanced antimicrobial efficiency targeting only the pathogenic micro organisms.**

Key words: *Pseudomonas*, Pyocyanin, phz A.

Pyocyanin compound produced by *Pseudomonas aeruginosa* is a biologically active secondary metabolite that functions as an inhibitory agent toward bacteria and fungi infecting plants, animals and human[1]. The mechanism by which pyocyanin inhibits bacterial and fungal species involves the arrest of electron transport chain[2]. The physiological significance of the pigment is not yet known but, it has been postulated that pigment production may give *Pseudomonas* aeruginosa a selective advantage in growth situations as a chelating agent[3].

The present study investigates on the production, purification, characterization and antifungal efficiency of pyocyanin. Further, the molecular characterization of pyocyanin was done to confirm the presence of particular pigment producing gene. Pyocyanin gene comprises seven operon site phz a, phz b, phz c, phz d, phz e, phz f and phz g having a total base pair of about 6720. Among the seven genes, phz a gene was amplified by PCR using suitable primer set (217 bp) and pyocyanin producing gene was confirmed[1].

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MATERIALS AND METHODS

Identification of bacterial strain

Fifty different clinical isolates of *P. aeruginosa* used were procured from Sharp laboratories, Perambur, Chennai. The bacterium was confirmed as *Pseudomonas* by its pigment production and standard biochemical reactions[4]. The pure culture of bacteria was maintained in Nutrient agar slants at 4ºC for experimental purpose.

Production and extraction of pigment

Soluble pigment of *P. aeruginosa* was produced by using nutrient broth after 24 hrs of incubation. Pigment production was confirmed by solvent extraction using chloroform which was further analyzed upon addition of 0.2N HCl[5].

Spectral Analysis of Pyocyanin pigment

Pigment was subjected to spectral analysis by three modes i.e. UV-spectrophotometer, Gas chromatography Mass spectroscopy (GC-MS) analysis[6,7] and Nuclear Magnetic Resonance (NMR) study[8,9].

Antifungal Activity Of Pyocyanin Compound

The antifungal activity of pyocyanin was determined by cross streak method toward *Candida albicans*, *C. krusei*, *C. glabrata*, *C. tropicalis* and *Cryptococcus neoformans*.

PCR Amplification of phzA gene

DNA was extracted from fresh cultures of *P. aeruginosa* strains incubated at 37 C over night onto nutrient agar by using PureFast® Bacterial Genomic DNA purification kit (HELINI Biomolecules, India), as per the manufacture instruction. Quality and Quantity of extracted DNA was asssed by 1% agarose gel. phzA Gene PCR was done by using the primers phzA - F Primer: 5'-TGAAGGGGGCCGAACGGTTACA-3' and phzA - R Primer: 5'-TCGCACTCGACCCAGAAGTGGT-3'. The primers were designed by using Primer3 software. *P. aeruginosa* pyocyanin biosynthesis operon (GenBank accession number AF005404.2). PCR reaction mixture comprised of 10× Taq reaction buffer, 2 mM $MgCl_2$, dNTP mix (10 mM each dNTP), primers (10pmol each), 2U of Taq DNA polymerase and PCR grade water to a final volume 25 µl. The PCR was thermocylced with initial denaturation at 94°C for 3 min followed by 35 cycles for 1min at 94°C, 1min at 60°C, 1 min at 72°C, and with final extension at 72°C for 5 min (Corpett research) the phzA Gene PCR amplicons were resolved in 2% agarose with ethidium bromide - 10 mg/ml by gel electrophoresis in 1x TAE buffer for ~40 min at 50V and analyzed by Gel documentation system. The expected amplicon size is 217 bp[1].

RESULTS AND DISCUSSION

Identification of Bacteria

Out of fifty clinical samples collected from laboratory, 42 isolates of *Pseudomonas* spp were producing pigment and confirmed based on Gram's staining, motility, cultural characteristic and by various biochemical reactions. Among 42 strains, 10 strains produced pigment and possessed antifungal activity toward various yeast species. These strains were further characterized.

Production and Extraction of pigment

Soluble compound was produced by using nutrient broth and extracted using chloroform as a solvent system which separates the blue color compound and it was confirmed further 0.2N HCl imparting pinkish red color that indicated the presence of pyocyanin pigment[5]. The characteristic feature of this organism is to produce soluble pigment namely pyocyanin and fluorescein. The pyocyanin compound was used in this study since it possessed antifungal activity with one hydroxyl phenazine possessing inhibitory effect[1].

Characterization of Pigment

Pyocyanin was further characterized by spectral analysis such as UV spectrophotometer, GC-MS and NMR which was already carried out and the pigment was confirmed by the mass and structural analysis[7,9].

Antifungal activity

The extracted and purified pigment was used to determine the antifungal activity against yeast species by cross streak method. Ten selected clinical isolates of *Pseudomonas aeruginosa* was tested against yeast species such as *Candida albicans*, *C. krusei*, *C. tropicalis*, *C. glabrata*, and *Cryptococcus neoformans* as shown in Table [2,9,10]. The tested yeast strains showed significant susceptibility toward pyocyanin and the presence of antimicrobial compound pyrrolnitrin was found responsible in arresting the electron transport chain[11].

Molecular study of pyocyanin producing gene

The pigment was initially screened by preliminary test and further confirmed genotypically by Polymerase chain reaction by determining the presence of pyocyanin biosynthesis gene (phz A), a protein among seven pyocyanin synthesis gene. All the 10 strains used in this study were subjected to gene amplification where an amplicon of 217bp was observed in all the ten samples confirming the phz A (Fig 1a, 1b) in comparison with the marker correlating with the work of Mavrodi *et al.,*[1]. This research work indicates that the secondary metabolite produced by *P. aeruginosa* possess antimicrobial activity wherein the bioactive compound derived from *Pseudomonas* can be structurally modified for an improved efficiency without any deleterious effect to the host targeting only the pathogenic micro organisms.

Table 1. Showing the antifungal efficacy of pyocyanin against yeast species

Yeast species	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
C. albicans	+	+	+	+	+	+	+	+	+	+
C. krusei	+	+	-	+	+	-	-	+	+	+
C. tropicalis	+	+	+	-	+	+	+	+	+	+
C. glabrata	+	+	-	-	+	-	-	+	+	+
C. neoformans	+	+	-	-	-	-	-	+	+	+

+ Inhibition of growth ; - No inhibition

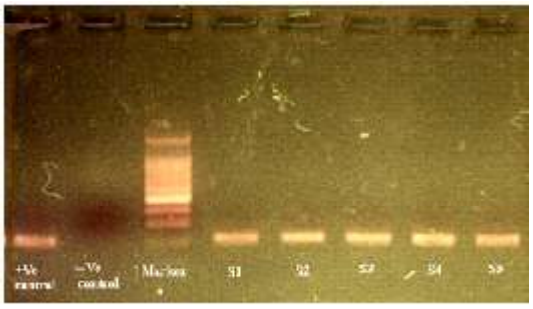

Fig. 1(a). Shows Gel picture of phz A amplified gene (217bp) of 5 strains of *P. aeruginosa* (S1-S5)

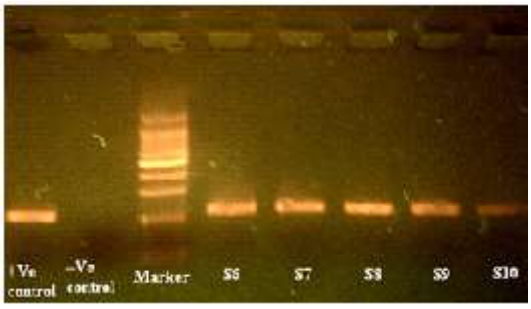

Fig. 1(b). Shows Gel picture of phz A amplified gene (217bp) of 5 strains of *P. aeruginosa* (S6-S10)

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