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Isolation of *Pseudomonas aeruginosa* for production of a remarkable antioxidant molecule pyocyanin

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Abstract

Pyocyanin, a major virulence factor produced by *Pseudomonas aeruginosa* is one of strongest free radical scavenging agent due to its redox active nature. It acts as antimicrobial agent against various pathogenic microbes. Thirteen isolates producing pyocyanin were isolated from environmental samples and clinical samples in order to exploit the antioxidant effect of pyocyanin. The higher producer of pyocyanin (HSS-6) was selected for further studies. The isolate was identified as *Pseudomonas aeruginosa* by 16SrRNA sequencing (GenBank Accession Number: MH038270) with 100% identity of gene sequencing data. The antioxidant efficacy of purified pyocyanin produced by *P. aeruginosa* MH038270 was evaluated as free radical scavenging agent by using DPPH assay. The purified pyocyanin shown to scavenge 74% DPPH and the IC_{50} value of the free radical scavenging activity of pyocyanin with DPPH assay was found to be 22.5µg/ml.

Keywords: Pyocyanin, *P. aeruginosa*, antioxidant activity, DPPH, antimicrobial.

Introduction

The production of pigment of microbial origin is now one of the emerging areas of research in order to investigate its potential for various industrial applications (Venil and Lakshmanaperumalsamy, 2009). *Pseudomonas aeruginosa* is common inhabitant of terrestrial and aquatic environment and can cause infection in a wide variety of organisms. It is able to grow in a normal atmosphere as well as in conditions deprived of oxygen (Green et al., 1974; Suthar et al., 2009). Pseudomonads are well known for the production of various secondary metabolites, resolved to survive under oxidative stress caused by environmental hazards and exhibit antagonism. An alarming rise of bacteria resistant to already existing antimicrobial compounds demands new strategies to seek effective agent against pathogenic microbes.

Since last five decades, phenazine compounds became great interest to clinical and pharmaceutical research (Laursen and Nielson, 2004). Phenazine compounds are biologically active secondary metabolites that function in microbial competitiveness and synthesized by *Pseudomonas* species (Mazzola et al., 1992). Phenazines are redox-active compound produced by these bacteria. These pigments are involved in virulence and iron acquisition (Dietrich et al., 2006). Pyocyanin is a water-soluble blue green, phenazine pigment synthesize by *P. aeruginosa* by Shikimic acid pathway. There are no other species of Gram-negative bacteria which produce pyocyanin, making its presence helpful in identification of organism. Pyocyanin exhibit activity similar to antibiotics towards many pathogenic microorganisms (Baron and

Rowe, 1981; Liang et al., 2008). This secondary metabolite has been studied intensively and drawn the attention of researchers for its broad spectrum properties. Pyocyanin has variety of potential biotechnological applications, as an antitumor agent (Laursen and Nielsen, 2004; Mavrodi et al., 2006) and the ability to inhibit the growth of pathogenic bacteria and fungi. Pigments produced by bacteria also have been applied for controlling biofilm.

The *P. aeruginosa* infections were difficult to treat because it develops multidrug resistance during the treatment of infection. In present study trial has been done to investigate the efficacy of pyocyanin pigment extracted from multidrug resistant *P. aeruginosa* as a potent antioxidant compound.

Materials and Methods

Sample collection

Various samples *viz;* soil sample, sewage sample, marine sample, slaughter house effluent sample and clinical samples have been collected (sterile plastic bags) from different location of Shimla, (H.P) India.

Isolation of Bacteria

The samples (soil sample, sewage sample, slaughter house effluent sample and clinical samples) were serially diluted (upto 10^{-8} dilutions) in the sterile physiological saline and 100µL suspension of each dilution was spread evenly on the Nutrient agar plates for primary isolation. The plates were incubated at 37^{o}C for 24-48h. Zobell's marine agar medium was used for the isolation of pyocyanin producing microorganism from marine samples.

Green colored colonies picked up from Nutrient agar plate and streaked on Cetrimide Agar plate. Cetrimide agar is a selective and differentiative medium for *P. aeruginosa*. It contains a quaternary ammonium compound known as cetrimide, which has broad spectrum bactericidal activity against a wide range of Gram- positive and some Gram- negative microorganism. The single colonies obtained on the plates were further streaked and the pure cultures obtained on Nutrient agar plate. Purity and identification of the *P. aeruginosa* was established by microscopic analysis, Gram's staining and biochemical test.

Screening, selection and identification of pyocyanin hyperproducer

A total number of 13 isolates were obtained from different sample and selected for further studies. Selected isolates were inoculated into nutrient broth and incubated for overnight at 37^{o}C in 200rpm orbital shaker to observe the production of pyocyanin pigment on the basis of visual assessment. The isolates which produce more pigment were selected to check the concentration of pigment. The centrifugation done at 10,000rpm for 10min to remove the cell pellets and the supernatant was used for assay method. Out of many microorganisms isolated, potent hyperproducer of pyocyanin pigment (HSS-6) was selected on the basis of amount of pigment produced. Identification of the potent producer of pyocyanin was carried out on the basis of morphological and biochemical characteristics. The isolate was sent to Eurofins Genomics Pvt. Ltd, Bangalore, India for identification by 16S rRNA sequencing. The isolate was identified as *P. aeruginosa* and used for further studies.

Production and extraction of pyocyanin

The optimized medium (pH 6.5) contained peptone 0.5 (%, w/v), beef extract 0.25 (%, w/v), NaCl 0.875 (%, w/v) and glycerol 2 (%, v/v) was used for the production of pyocyanin. The flasks were incubated at 37^{o}C for 120h in orbital shaker at 50rpm. Pyocyanin was extracted from culture supernatant and measured on the basis of absorbance of pyocyanin in acidic solution at 520nm (Essar et al., 1990). Concentration, expressed as micrograms of pyocyanin produced per milliliter of culture supernatant, were determined by multiplying the optical density at 520nm (OD_{520}) by 17.072 (Sarkisova et al., 2005).

DPPH assay to assess the antioxidant activity of pyocyanin

DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in methanol. This is a simple method used to determine the antioxidant activity, utilizes the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical (Liyana and Shahidi, 2005). The odd electron in the DPPH free radical gives a strong absorption maximum at 517nm and is purple in color. The color turns from purple to yellow as the molar absorptivity of the DPPH radical at 517nm reduces when the odd electron of DPPH radical becomes paired with hydrogen from a free

radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolorization is stoichiometric with respect to number of electrons captured.

Purified pyocyanin pigment from *P. aeruginosa* MH038270 of various concentrations (3-36µg/mL) were mixed in 1mL of freshly prepared 0.5mM DPPH methanolic solutions and final volume adjusted to 2mL with methanol. The resulting solutions were incubated in dark at 37°C for 30 min and A_{517} values were recorded. Lower A_{517} values represented higher DPPH scavenging activity. The % radical scavenging activity of pigment was calculated from the decrease in absorbance in comparison to the control using the following equation:

Free radical scavenging activity (%) =

$$A_{control} - A_{sample} / A_{control} \times 100$$

$A_{control}$ is absorbance of the DPPH + methanol
A_{sample} is absorbance of free radical solution with pyocyanin.

Ascorbic acid of same concentration was used as standard. The results were statistically analyzed for three independent repeats.

Results and Discussion

Isolation and identification of pyocyanin producing *P. aeruginosa*

The result of pyocyanin producing isolates has been presented in Table.1. Total 13 isolates shown the production of green color pigment on Nutrient agar plates. Out of 13 positive isolates, isolate HSS-6 with maximum pigment production was selected for further studies.

Table.1: Isolation of pyocyanin producing *P. aeruginosa* from different samples

S.No.	Location	Isolate name	Pigment production
Soil Samples			
1	Laalpaani, Shimla	-	ND
2	ITH (HPU)	-	ND
3	HPU canteen	-	ND
4	Vipasha Hostel (HPU)	-	ND
Slaughter house samples			
5	Effluent sample, Laalpaani, Shimla	SSH-1	+
		SSH-2	+
		SSH-3	+++
6	Soil sample, Rohru	RSH-1	+
Marine samples			
7	Vishakapatnam	-	ND
8	Mumbai	-	ND
Sewage sample			
9	Pink petal cafe (HPU)	PPS-1	+
		PPS-2	+
10	Vipasha hostel (HPU)	-	ND
11	HPU canteen	-	ND
12	ITH (HPU)	-	ND
Clinical samples			
13	IGMC, Shimla	HSS-1	+
		HSS-2	+
		HSS-3	++
		HSS-4	+
		HSS-5	+
		HSS-6	+++
		HSS-7	+

(ND: Not determined)
(Visual assessment of pyocyanin amount (blue pigment): + low, ++ moderate, +++ high)

The isolated organism was identified as *P. aeruginosa* on the basis of colony morphology, cultural characteristics and 16SrRNA sequencing (Fig.1).

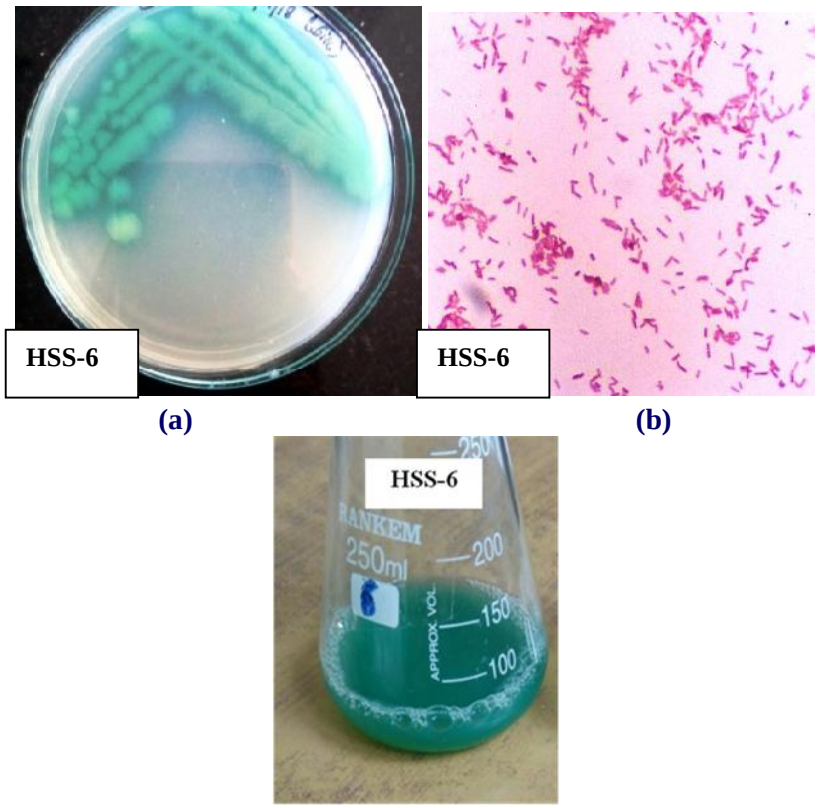

Fig.1. Growth of HSS-6 on NA plate (a), Microscopic view (1000X) (b) and Production of pigment in Nutrient Broth inoculated with HSS-6 (c).

Blue-green soluble pigment namely pyocyanin production was indicated by change in color of nutrient broth and the addition of 0.2N HCl, the extracted pigment change its color to red pink from blue confirmed the presence of pyocyanin.

According to the results of 16S rRNA nucleotide sequence analysis, the bacterium was identified as *Pseudomonas aeruginosa* and a phylogenetic tree was constructed by the neighbor-joining method (Fig.2). The nucleotide sequence of the culture (HSS-6) was deposited at GenBank and given with the GenBank accession number and MH038270 (HSS-6).

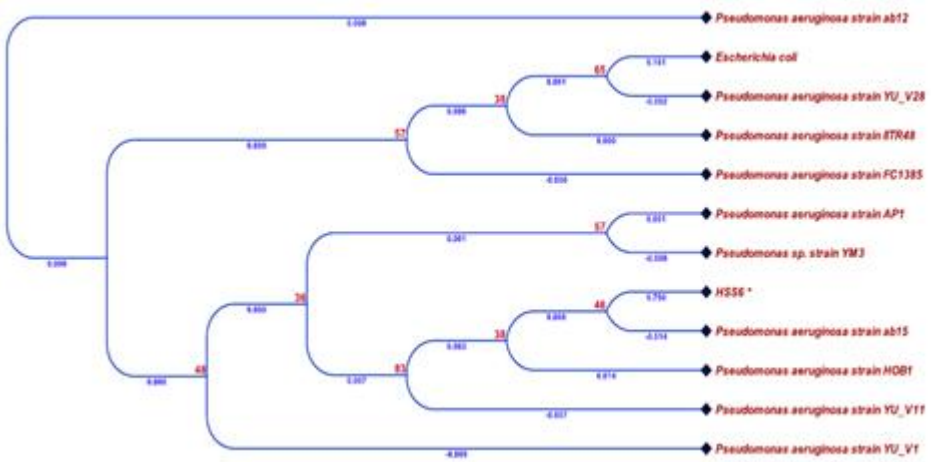

Fig.2. Phylogenetic tree of isolated *P. aeruginosa* HSS-6

(Bootstrap values are indicated at the branch points. The bar indicates a branch length equivalent to 0.02 changes per amino acid. Phylogenetic tree was constructed by the neighbor-joining method, using MEGA software, version 5.2)

Antioxidant activity of pyocyanin

The pigments were known to have antioxidant activity. An antioxidant is molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that can produce free radicals, leading to chain reactions that may damage cells. Free radical scavenging activity or antioxidant activity of pyocyanin was estimated by DPPH radical scavenging assay (Liyana and Shahidi, 2005). The antioxidant property of the pyocyanin of *P. aeruginosa* was evaluated. Ascorbic acid was used as standard or the positive control. The free radical scavenging activity of pyocyanin was dose dependent and maximum activity was found to be 74.9% at 30µg/mL of pyocyanin concentration (Fig.3). The IC_{50} value obtained was 22.4µg/mL. It has been reported that pyocyanin produced from *P. aeruginosa* BTRY1 strain has higher free radical scavenging activity (80%) at 0.2µg/mL, a concentration much lower than that of ascorbic acid used (Laxmi and Bhat, 2006). The high antioxidant activity at very minute concentration of pyocyanin, is a positive indication which suggests that this product is safe to use (Liyana and Shahidi, 2005). Chandran (2014) evaluated the antioxidant activity of pyocyanin pigment which was found to be 55% at 500µg/mL concentration. Dahah et al., (2016) reported that the IC_{50} value of the pyocyanin was 3.15µg/ml, as compared to that of (IC_{50} 7.79µg/ml) obtained with ascorbic acid, a well-known antioxidant. The pyocyanin from *P. aeruginosa* CGR-3 showed free radical scavenging activity of about 38% at 4.25 µg/mL of pyocyanin concentration (Rani et al., 2018).

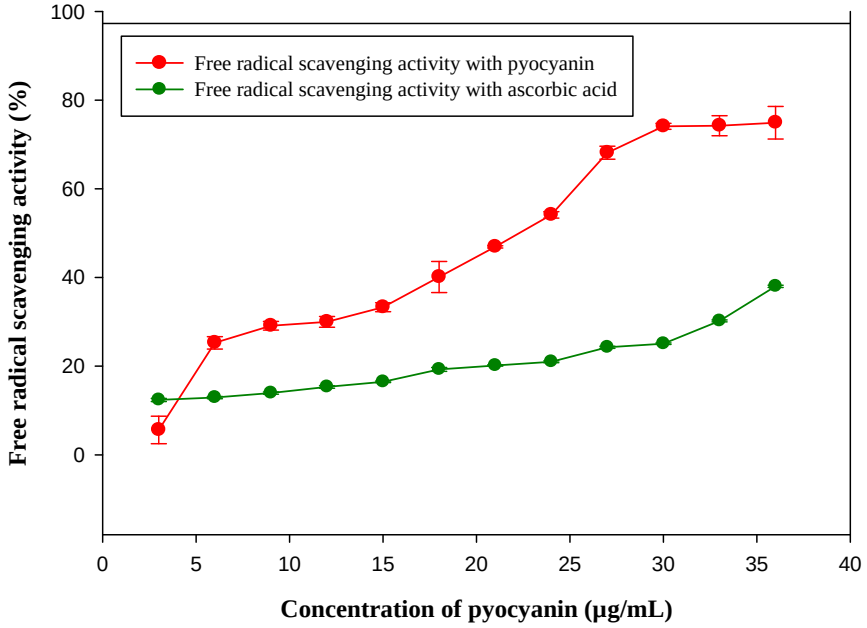

Fig.3. Free radical scavenging activity of pyocyanin from *P. aeruginosa*

Conclusion

This study revealed that the pyocyanin produced by isolated *P. aeruginosa* has a powerful antioxidant activity even at very low concentration as compared to standard. The pyocyanin pigment can becomes a valuable new addition to the currently existing antimicrobial drugs to help in the prevention of various infections caused by pathogenic microorganism. The applications of pyocyanin at very low concentration do not show any pathological effects in eukaryotic system and can be used as biocontrol agent against fungal and bacterial pathogens.

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Conflict of interest

The authors have no financial conflicts of interest to declare.

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