

Original Research Article

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## Production, Characterization and Cytotoxic Evaluation of Pyocyanin Pigment Extracted from *Pseudomonas aeruginosa* Isolated from Industrial Soil Resources

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### ABSTRACT

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Thirty-five isolates belonging to the various genus of bacteria family were isolated from 25 different soil samples from various industrial areas from different districts of Tamil Nadu. One out of them were identified as *P.aeruginosa* (P1) and screened for pyocyanin production using submerged fermentation. The Pyocyanin production reached  $\mu\text{g/ml}$  on modified pseudomonas broth. The identification of strain was confirmed by 16s rRNA, the similarity with other strains available in the database was 98% (FDAARGOS\_767). P1 was accessed at gene bank with accession number CP041008.1. Identification of the PhzM gene encoding PhzM enzyme was carried out using gene-specific primer followed by PCR. 190bp size gene was amplified using PCR. PhzM gene was confirmed and similarity with (FDAARGOS\_767) strain was 100% in the database. Pyocyanin was extracted by standard chloroform extraction method, purified by HPLC (C17 column) and characterized by UV-Vis absorption spectroscopy, Particle size, zeta potential, FT-IR and LC-MS. The SEM analysis of pyocyanin pigment showed the particle size of microencapsulated pyocyanin. The cytotoxic effect of pyocyanin was investigated using VERO cells and it showed a concentration-response relationship between the concentration and cell viability the cytotoxicity increased gradually with the increase of its concentration.

## Introduction

*Pseudomonas aeruginosa* is a gram-negative, aerobic rod shaped bacterium, ubiquitous organism in nature and widespread in soil, water and many other environment. *Pseudomonas* spp. producing a variety of extracellular phenazine pigments. *P. aeruginosa* was a common environmental gram-negative *Bacillus* type microbe (Budzikiewicz, 1993).

It was an opportunistic human pathogen as well, was known for its ability to produce pigments. *P. aeruginosa* was widely distributed in the environment; it was founded in soil, water, skin flora, and most man made environments throughout the world, and had thus colonized many natural and artificial environments (Kerr, 1999).

Majority of *P.aeruginosa* produce Pyocyanin pigment which was also called as “blue pus”. Pyocyanin is a water soluble blue green phenazine nitrogen-containing heterocyclic compound. Pyocyanin is redox active secondary metabolite. It is an extracellular pigment which is produced by *Pseudomonas aeruginosa* (Lyczak *et al.*, 2000).

Pyocyanin production is abundant in medium with low iron content and plays an important role in iron metabolism. The presence of pyocyanin is easy to detect due to its blue color that turns stationary phase cultures of *P.aeruginosa* into green color. It has various pharmacological effects on prokaryotic and eukaryotic cell and also used to control phytopathogen. Pyocyanin is a zwitterion at blood pH, it is easily able to cross the cell membrane (Lau *et al.*, 2004).

Pyocyanin usually exists in three different forms oxidized, monovalently reduced or divalently reduced. Mitochondria aids in the conversion of pyocyanin between its redox

states, Pyocyanin generates reactive oxygen species and it is because of its redox active state. Pyocyanin generally inactivates Catalase enzyme by reducing the gene transcription and targeting the enzyme. Pyocyanin modulates glutathione which is one of its important characteristics. In particular the pool of the reduced form is depleted while the oxidised form is promoted by hydrogen peroxide which is not mutated by Catalase (O'Malley *et al.*, 2004).

A variety of redox active phenazine compound are produced by strain of *P.aeruginosa*, including pyocyanin, phenazine 1- carboxylic acid and phenazine 1- carboxamide (Ran *et al.*, 2003). The phenazine based pyocyanin has the ability to generate reactive oxygen species (ROS). It has great role in biosensors as a redox compound for carrying out electron transfer between enzyme molecules and the electrode material (Priyaja, 2013).

The objectives of this study were 1) to isolate pigment producing *P.aeruginosa* from different soil samples; 2) purify and characterize pyocyanin by standard protocol and techniques; 3) to study the functional activity of pyocyanin.

## Materials and Methods

### Sample collection and processing

Twenty five soil samples were collected at a depth of 15 cm from different industrial areas at different districts of Tamil Nadu, India and kept in sterile polyethylene bags at 4°C until isolation process. Sampling procedures and microbial analysis were carried out according to standard methods (AOAC, 2016). Fifty grams of each soil sample was placed in a 100ml sterile PBS (1X) solution and shaken at 250rpm for 2 hrs. 1ml of soil suspension was serially diluted up to 10<sup>9</sup> dilution. 10<sup>7</sup>, 10<sup>8</sup>,

10<sup>9</sup> dilutions were plated onto nutrient agar and pigmented colonies were selected and primarily identified by standard biochemical tests including; oxidase, Catalase, nitrate reduction, citrate utilization (O'Malley *et al.*, 2004).

### **Molecular identification of *P.aeruginosa* strain and *PhzM* gene**

The genomic DNA was extracted from the overnight culture using DNeasy® Blood and Tissue kit (Qiagen) as per the manufacturer's instructions and quantified in Nanodrop One (Thermoscientific, USA). The PCR was carried out using 16S rRNA universal primers in a thermal cycler (Veriti, Applied Biosystems). PCR mixture consists of Amplicon master mix 12.5 µl, 1.0 µl each primer, 3.0 µl of DNA and 7.5 µl of nuclease free water. The cycle conditions were initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1.3 min and final extension at 72°C for 5 min (Hoshino, 2011). 10 µl of PCR mixture was loaded on to 0.8% agarose gel to examine the PCR product against 1Kb ladder (Thermofisher). The PCR products were purified using MinElute® Gel extraction kit (Qiagen) as per the manufacturer's instructions. The gel eluted PCR products were sequenced (Eurofins, Bangalore). The sequences obtained were then analysed for their homology with the gene sequences in the Genbank. The gene sequences flanking the entire ORF of the selected gene of *PhzM* were downloaded from NCBI, GenBank (Accession number CP041008.1). Primers used for identifying gene were designed using primer 3 plus software. Polymerase Chain Reaction amplifications were performed using the designed primers. The annealing temperatures of the primers were optimized by gradient PCR. PCR mixture consists of Amplicon master mix 12.5 µl, 1.0 µl each

primer, 3.0 µl of DNA and 7.5 µl of nuclease free water. The cycle conditions were initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 45 sec, extension at 72°C for 1 min and final extension at 72°C for 7 min (Onbasli and Aslin, 2008). 10 µl of PCR mixture was loaded on to 2% agarose gel to examine the PCR product against 100 bp ladder (Thermofisher). The 16S r RNA primers used in this study were Forward: 5'AGATTTGATCCTGGCTCAG3'; Reverse: 5'GGTTACCTTGTTACGACTT3' and *PhzM* gene specific primers used in this study were: Forward:5'GCTGCGGTAATTTGATAACA3 Reverse: 5'AGATCTCGAAGGCCACCAG3'

### **Pyocyanin pigment production**

*P.aeruginosa* was cultured in 1 litre conical flask containing modified pseudomonas broth containing the following ingredients according Deziel *et al.*, 2004. 20 g of dextrose (Sigma Aldrich), 20 g of peptone (Himedia), 10 g of potassium sulphate (SRL), 50 ml of glycerol, 4g of pseudomonas broth (Himedia) and 1.4g of magnesium Chloride (SRL). The organism was inoculated at the rate of 10<sup>9</sup> cells and incubated for 2-3 days at 35°C under shaking environment. The change in color of the pigment to bluish green indicated the pigment production (Sudhakar *et al.*, 2013).

### **Extraction of pyocyanin**

The change in color of the pigment to bluish green indicated the pigment production of pseudomonas. After 3 days of incubation broth was centrifuged (Thermo fisher cooling centrifuge) at 8000 rpm for 10 minutes. The culture supernatants were transferred into conical flasks and extracted with chloroform (1:2) and the aqueous phase was removed. The bottom layer was re extracted with 1 ml of 1 N HCl until color change was observed. The extract was fed into rotary vacuum

evaporator (SARE-T43 Model, SPAN Automation) and heated upto 70°C at 80 rpm for 2 hrs to concentrate the pigment and remove ethanol from pigment. The concentrated pigment solution was used as a source of pyocyanin. Pyocyanin was quantitatively assay based on measuring the absorbance of pyocyanin in the acidic form at 520 nm according to the following equation (Essar *et al.*, 1990).

$$\text{Concentration of pyocyanin } (\mu\text{g/ml}) = \text{O.D}_{520} \times 17.072$$

### **Purification and characterization of pyocyanin**

The crude pyocyanin pigment stored in sterile containers at 4 °C was re-dissolved in 10 ml of chloroform and purified using HPLC separation module Alliance 2695 with diode-array detector PDA 2996 (Waters, Millford, USA) were used. Detection was carried out at the wavelength 275 nm. Separation was performed on a chromatographic column Biorad 300SEC-70 10x300 mm, 3 µm particle size (California, USA). Linear gradient and flow rate 2 ml/min were used. Mobile phase A consisted of water/Methanol/trifluoroacetic acid (90:5:5) and mobile phase B acetic acid/water/trifluoroacetic acid (5:90:5). The column temperature was set at 47°C and injection volume was 10 µl. Data was set, observed and evaluated by software Empower (Waters, Millford, USA). Purified pyocyanin was eluted with 25% methanol in chloroform. The eluted fractions were examined by scanning UV-Vis spectrophotometer and fractions having the same λ-max were collected together. The purified pyocyanin was subjected to spectroscopic analysis. Ultraviolet and visible absorption spectra of purified pyocyanin dissolved in chloroform or 0.1 N HCl was recorded over a range of 200–700 nm. The Bruker IFS66v FT-IR instrument (VERTEX model, Bruker optics, Germany)

consists of globar and mercury vapor lamp as sources, an interferometer chamber comprising of KBr (Potassium Bromide) and Mylar beam was used for determining the functional groups. Liquid chromatography-diode array detection-mass spectrometry (LC-DAD-MS) was performed on an Agilent eclipse HP 1100 LC system with a DAD and a 5 µ XDB C18 column (Phenomenex, Torrance, CA). SZ100 nano partica (Horiba) instrument was used for analyzing particle size and zeta potential of the pigments.

### ***In vitro* bioassay (Cytotoxicity)**

Vero cells were sub cultured and split as per the cell ratio. Pigments were diluted into five or ten fold dilution. Each concentration was added in triplicate. Pigments were treated for a defined time period (1-4 hrs). At the end of exposure pigments were removed from the wells and 200µl of fresh medium was added along with 50µl of MTT was added to all the wells. Plates were wrapped in aluminum foil and incubated at 37°C for 4 hours. After incubation medium with dye was removed and the formazan crystals were dissolved by adding 200µl DMSO. Glycine buffer was added to adjust the pH. Absorbance was read at 570nm (Khan *et al.*, 2012).

### **Results and Discussion**

#### **Isolation of *P.aeruginosa* from different soil samples**

*Pseudomonas aeruginosa* is a full lineage bacterium; *Proteobacteria* class: *Gammaproteobacteria*; Order: *Pseudomonadales*; Family: *Pseudomonadaceae*; Genus: *Pseudomonas*; Species: *aeruginosa* (Hasset, 1996). *P. aeruginosa* is a Gram-negative, aerobic (facultative anaerobic), rod-shaped bacterium with unipolar motility. It was identified as an opportunistic pathogen in human. When

oxygen, nitrate, and nitrite are absent, it is able to ferment arginine and pyruvate by substrate-level phosphorylation (Richard *et al.*, 2008). Soil samples were widely collected from 25 district industrial area from which 35 different bacterial isolates were identified and pigment producing *P.aeruginosa* (Figure 1) was isolated from soil sample collected from Guindy Industrial estate, Chennai.

The isolated organism was cultured on nutrient agar and observed its biochemical and sugar fermenting ability. Isolated organism was found to utilize dextrose, fructose, trehalose, mannose and glucose (Table 1). *P.aeruginosa* was found to be a gram -ve rod shaped non - endospore forming motile bacterium which was positive for Catalase, oxidase and other biochemical activity (Table 2). The isolate could produce blue-green pigment on nutrient medium and developed yellow-green fluorescent pigment under UV light (520 nm). Identification was confirmed by molecular tools and extracted pigments were purified and characterized.

### **Molecular identification of *P.aeruginosa* and its *PhzM* gene**

The 16S rRNA sequence has been used as taxonomic standard for the phylogenies of bacterial species. The 16S rRNA gene sequence depicts the differentiation between organisms at the genus level in major phyla of bacteria. The identification of *P.aeruginosa* strain was confirmed by 16s rRNA, the similarity with other strains available in database was 98% (FDAARGOS\_767). P1 was accessed at gene bank with accession number CP041008.1. Identification of *PhzM* gene encoding PhzM enzyme was carried out using gene specific primer followed by PCR. 190bp (Figure 2) size gene was amplified using PCR. *PhzM* gene was confirmed and similarity with (FDAARGOS\_767) strain was 100% in database.

### **Purification and characterization of Pyocyanin**

The crude pyocyanin (Figure 3) of *P.aeruginosa* strain was purified using High performance liquid chromatography (C17 column). Pyocyanin was eluted with 20% methanol in chloroform. The blue color fraction were collected, dried and stored in -20°C for characterization. The physicochemical properties of purified Pyocyanin revealed that the pigment was readily soluble in chloroform, HCl. Various other solvents like benzene, hexane, ethanol, diethylether were used to extract pyocyanin from the broth. Complete extraction of pyocyanin was done using chloroform and benzene as they were more efficient in extraction of pyocyanin. The concentration of Pyocyanin was 5.98µg/ml.

The absorbance spectrum of purified Pyocyanin was estimated from 200nm to 800nm using UV-Vis spectrophotometer. The dried Pyocyanin was dissolved in methanol and absorption was estimated. The absorption was maximum at 300, 450 and 730nm (Figure 4). These results were in accordance to the previous research findings of Priyaja, 2013 studied the UV-Vis spectrum of pyocyanin and found its peaks at 201nm, 320nm and 718nm.

The particle size and zeta potential of the pyocyanin was determined using nano particle instrument. The purified Pyocyanin had a particle size of 107.4 nm (Figure 5) and the zeta potential of the purified Pyocyanin was 19.2 mV (Figure 6). This result was in acceptance to the previous research findings of Lau *et al.*, (2004).

This dried pigmented filtrate was subjected to High performance liquid chromatography to obtain colored metabolite containing fractions. The fractions obtained were subjected to LCMS after combining them.



Fraction 1-4 was found to contain an intense peak of m/z 477 which when subjected to LCMSMS showed the fragments with m/z

478 and 354 which correspond to the structure of Pyocyanin (Figure 7).

**Table.1** Sugar fermentation ability of *P.aeruginosa*

S.No	Sugars	<i>Pseudomonas aeruginosa</i>
1	Lactose	-
2	Xylose	-
3	Maltose	-
4	Fructose	+
5	Dextrose	+
6	Galactose	-
7	Raffinose	-
8	Trehalose	+
9	Melibiose	-
10	Sucrose	-
11	L-Arabinose	-
12	Mannose	+
13	Inulin	-
14	Sodium Gluconate	-
15	Glycerol	-
16	Salicin	-
17	Dulcitol	-
18	Sorbitol	-
19	Mannitol	-
20	Adonitol	-
21	Arabitol	-
22	Erythritol	-
23	A-Methyl-D-Glucoside	-
24	Rhamnose	-
25	Cellobiose	-
26	Melezitose	-
27	A-Methyl-D-Mannose	-
28	Xylitol	-
29	D-Arabinose	-
30	Glucose	+
31	Sorbose	-
32	A-Methyl-D-Glucoside	-

**Table.2** Biochemical tests of *P.aeruginosa*

S.No	Biochemical test	<i>Pseudomonas aeruginosa</i>
1	Gram staining	Gram –ve rod
2	Motility test	Motile
3	Endospore staining	Negative
4	Oxidase test	Positive
5	Catalase test	Positive
6	Methyl red test	Negative
7	Urease test	Positive
8	Indole test	Negative
9	Voges proskeur’s test	Negative
10	ONPG	Negative
11	Esculin Hydrolysis	Positive
12	Malonate Utilization	Positive
13	Nitrate reduction	Positive
14	Lysine utilization	Negative
15	Ornithine utilization	Negative
16	Phenylalanine deamination	Negative
17	Citrate test	Positive
18	H <sub>2</sub> S production	Negative

**Table.3** Data represent the mean±standard error of the values

S.No	Control	50mg/ml	40mg/ml	30mg/ml	20mg/ml	10mg/ml
CC <sub>50</sub> Values	0.153	0.0956	0.117	0.133	0.139	0.141
% vialibilty	100±0.01	62.40±0.01	76.52±0.01	86.96±0.01	91.09±0.01	92.40±0.01

**Figure.1** *Pseudomonas aeruginosa* streaked on nutrient agar medium plate

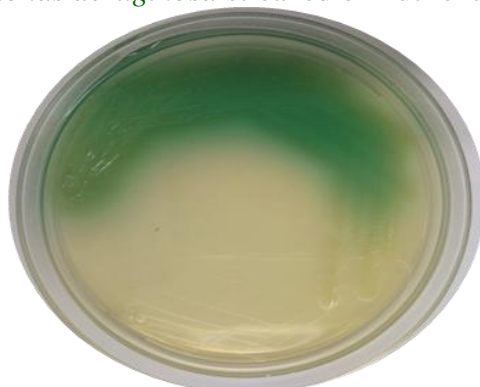


Figure.2 2% Agarose gel showing PCR amplicon of *PhzM* primer

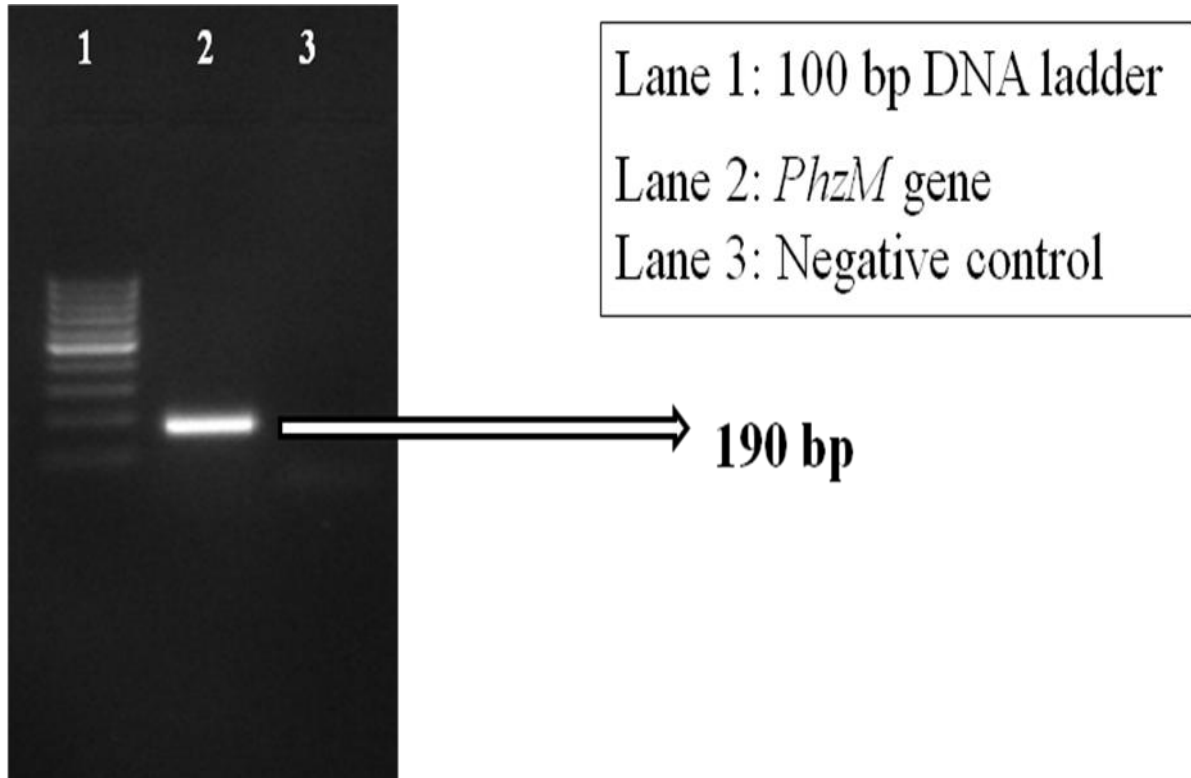
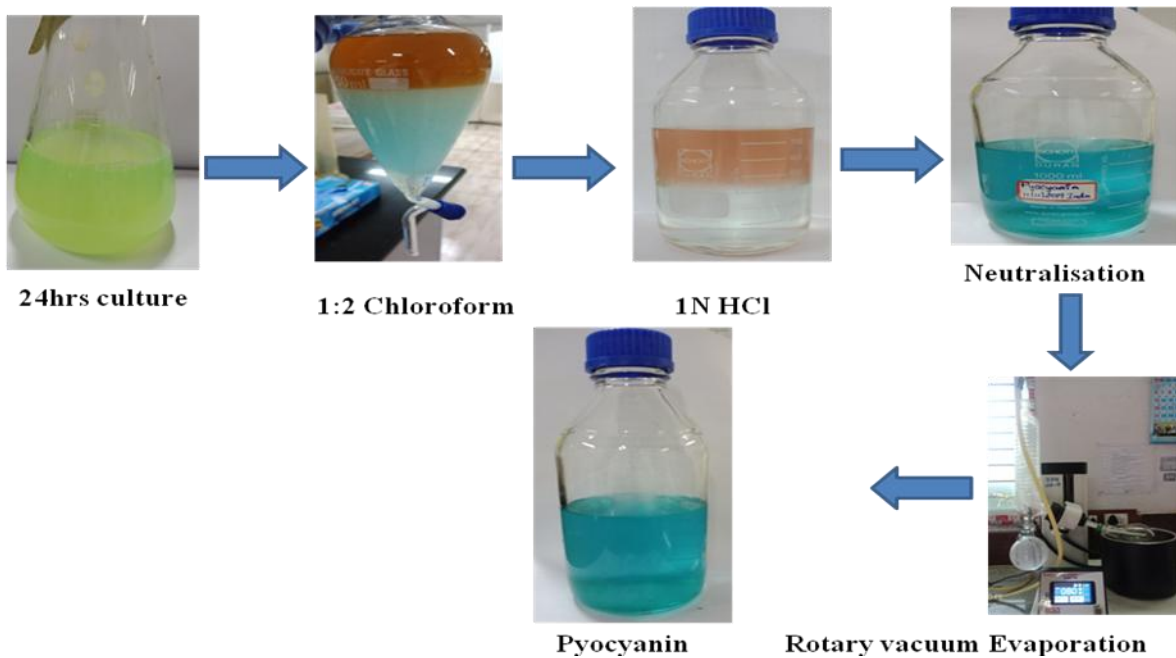
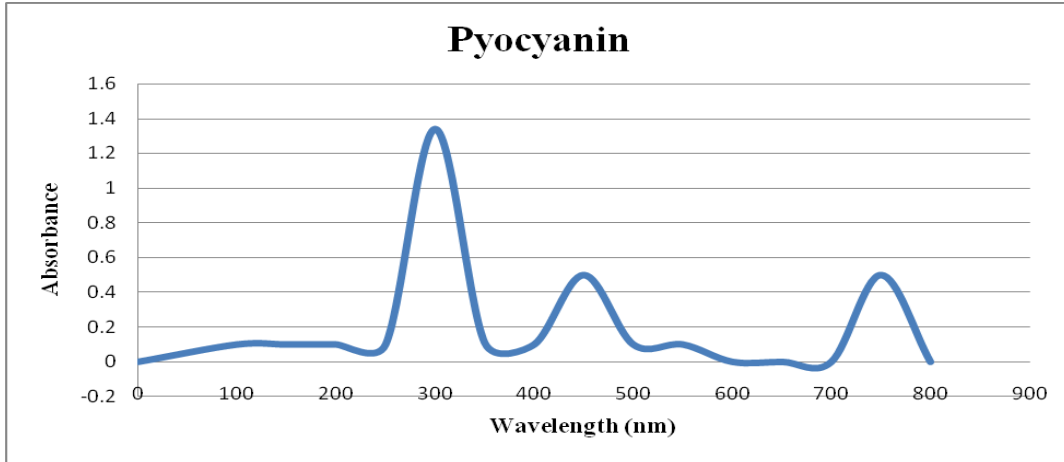


Figure.3 Extraction of crude Pyocyanin pigment

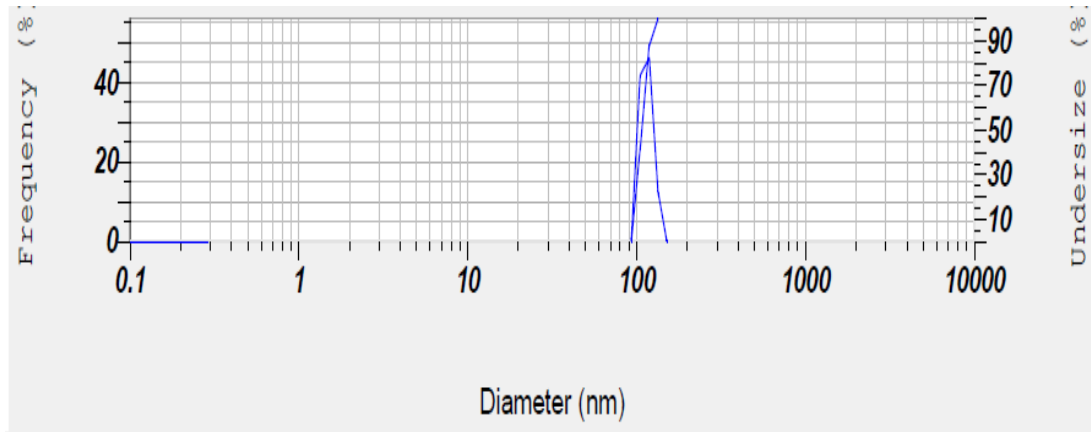




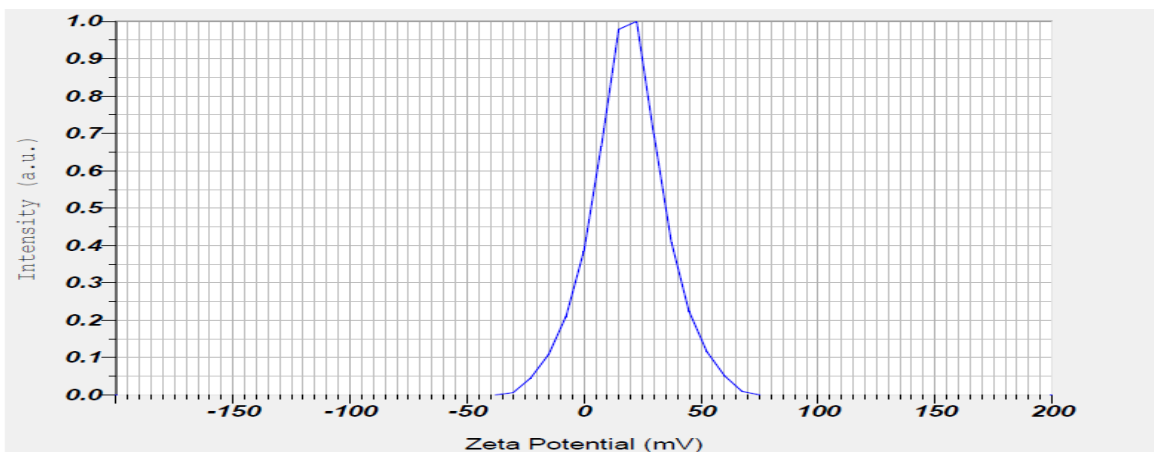
**Figure.4** UV-Vis absorption spectrum of purified pyocyanin dissolved in methanol



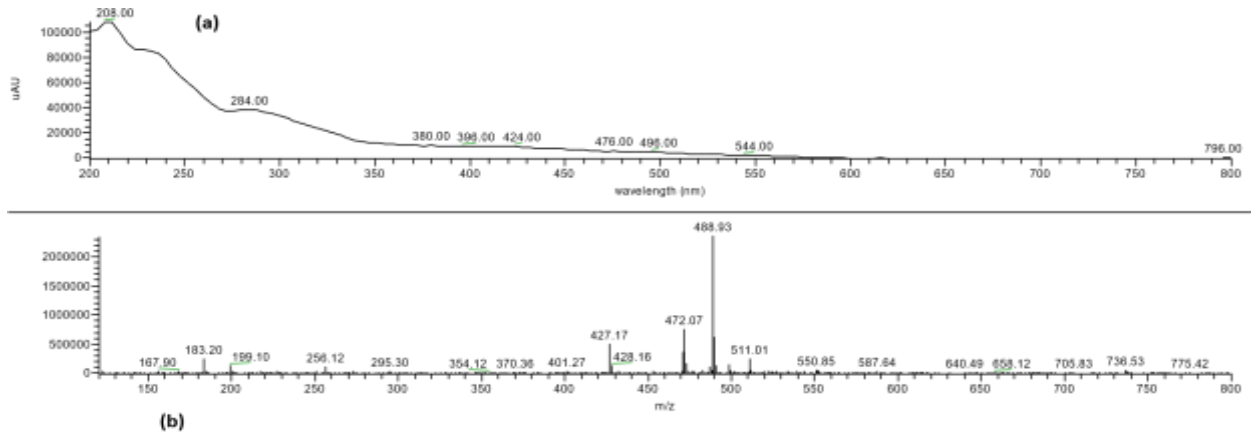
**Figure.5** Particle size of purified pyocyanin dissolved in methanol



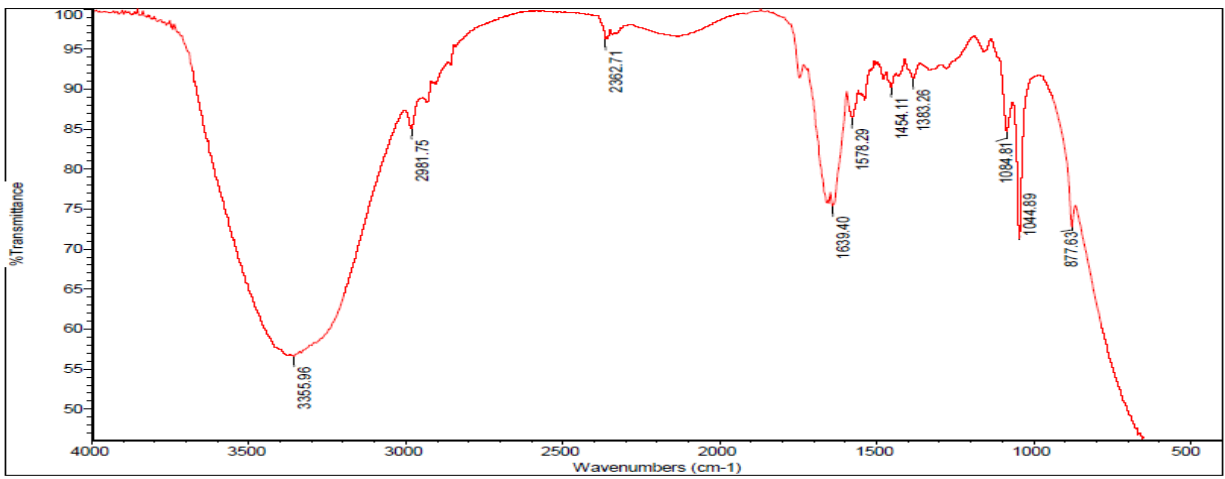
**Figure.6** Zeta potential of purified pyocyanin dissolved in methanol



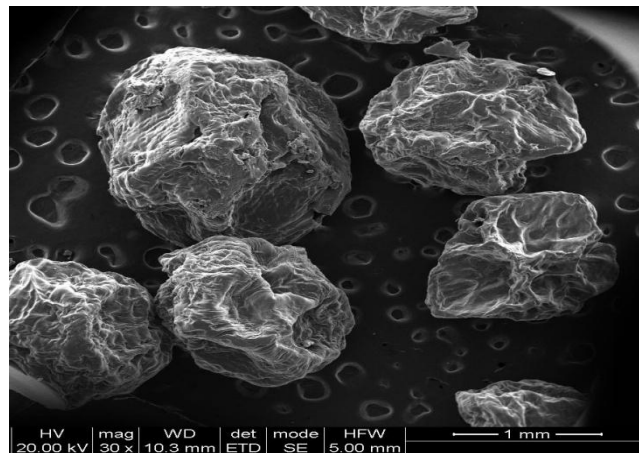
**Figure.7** Fragmentation pattern of Pyocyanin (b) (Fraction1-4) (showing an intense peak of m/z 477 and the fragments m/z 478 and 354 in LCMS)



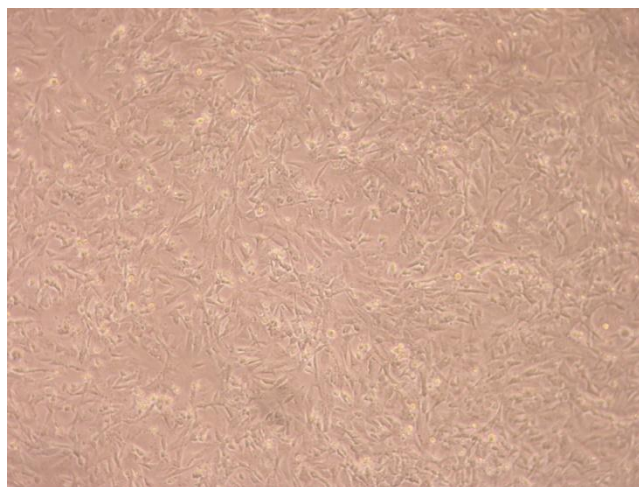
**Figure.8** FT-IR spectrum of purified Pyocyanin



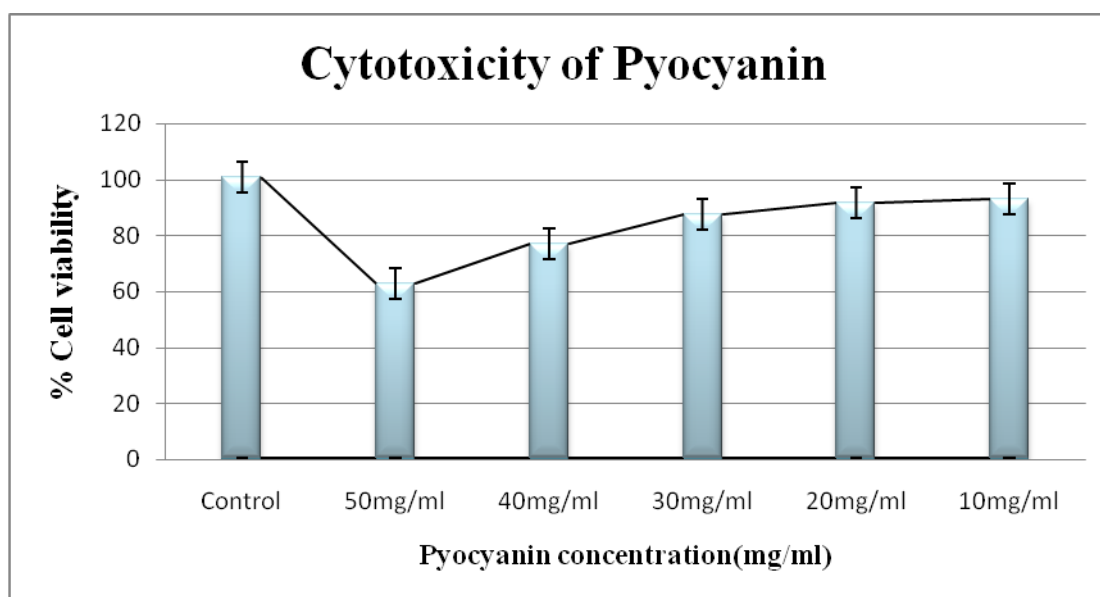
**Figure.9** SEM micrographs of pyocyanin



**Figure.10** VERO cells treated with Pyocyanin viewed under inverted microscope



**Figure.11** Cytotoxicity of pyocyanin towards cell lines using MTT method



FTIR spectroscopy of the methanolic pigments filtrate was done in order to detect chemical classes of compounds based upon their functional groups present in the cultural filtrate (Fig. 8). In pyocyanin, a peak at 877.63cm<sup>-1</sup> showed C=O stretch indicating presence of pyrone of coumarin ring and peaks at 1578.29 cm<sup>-1</sup>, 1639.40 cm<sup>-1</sup> corresponded to benzene ring's C=C stretch. There was C-H bending peak at 1454.11 cm<sup>-1</sup> and 1383.26cm<sup>-1</sup> respectively. C=O

stretching band at 1044.89cm<sup>-1</sup>, 1084.89cm<sup>-1</sup> and 635cm<sup>-1</sup> in the culture filtrate indicated the presence 1,4-quinones of anthraquinones respectively. The bands at 3355.96 cm<sup>-1</sup>, 2362.71 cm<sup>-1</sup> and 2981.75cm<sup>-1</sup> corresponded to C-H stretch which has been reported in some alkaloids and hydrogen bonded OH moiety.

The microstructure of Pyocyanin was observed using Scanning Electron

Microscope (SEM). Examination of the SEM micrographs showed that the particle size of the dried Pyocyanin ranged 100µm to 1mm. The particle was observed to be in irregular spherical shape with cavities and agglomeration. The agglomeration occurs due to the static electrical force (Figure 9).

### ***In vitro* bioassay (Cytotoxicity)**

The reduction of MTT ([3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide]) in the cell assesses the functional intactness of mitochondria based on the enzymatic reduction of the tetrazolium salt by the mitochondrial dehydrogenase in viable cells (Denizot and Lang 1986).

MTT and neutral red are probably the most commonly used colorimetric indicators of cell viability and they have been used to evaluate cytotoxicity in a quantitative way in contrast with cell morphology evaluation by inverted light microscopy which is qualitative and more subjective (Smee *et al.*, 2002).

The toxicity of pyocyanin to Vero cells (Figure 10) was investigated by the colorimetric MTT assay. This cytotoxicity was also verified with the cell lines used in this study, and Table 3 shows the % cell viability values obtained with pyocyanin. Pyocyanin showed a concentration-response relationship since the cytotoxicity increased gradually with the increase of its concentration (Figure 11). The % cell viability was calculated using the formula.

% cell viability = (Corrected abs. of the sample/Corrected abs. of the control)\* 100

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