



## Confocal and SEM imaging to demonstrate food pathogen - biofilm biocontrol by pyocyanin from *Pseudomonas aeruginosa* BTRY1

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**Abstract:** An assortment of redox-active phenazine compounds like pyocyanin with their characteristic blue-green colour are synthesized by *Pseudomonas aeruginosa*, Gram-negative opportunistic pathogens, which are also considered one of the most commercially valuable microorganisms. In this study, pyocyanin from *Pseudomonas aeruginosa* BTRY1 from food sample was assessed for its antibiofilm activity by micro titer plate assay against strong biofilm producers belonging to the genera *Bacillus*, *Staphylococcus*, *Brevibacterium* and *Micrococcus*. Pyocyanin inhibited biofilm activity in very minute concentrations. This was also confirmed by Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM). Both SEM and CLSM helped to visualize the biocontrol of biofilm formation by eight pathogens. The imaging and quantification by CLSM also established the impact of pyocyanin on biofilm-biocontrol mainly in the food industry.

**Key words:** Pyocyanin; Antibiofilm activity; SEM; CLSM

### Introduction

*Pseudomonas aeruginosa* are Gram-negative, aerobic rod-like bacteria, motile by single polar flagellum [1, 2]; thriving in normal atmospheric as well as hypoxic atmospheres, besides colonising many natural and artificial environs [3, 4, 5]. They attract attention owing to the different pigments produced, like pyocyanin (blue-green), pyoverdinin (yellow, green and fluorescent), pyomelanin (light-brown) and pyorubrin (red-brown) [6, 7]. Nearly 90–95 % *P. aeruginosa* isolates produce pyocyanin, usually referred to as “blue pus” (from pyocyanus) [8, 9,10].

Recent research indicates that secretions from *Pseudomonas aeruginosa* also inhibit biofilm formation by several fungi, as well as other bacterial pathogens [11]. There are several reports that *P. aeruginosa* itself can inhibit growth and biofilm formation [12]. Bacterial pigments used for biofilm control are reported to have free radical scavenging activity along, which in addition to absence of cytotoxicity, makes them useful in food industry for control of food borne infections. Here, the antibiofilm activity of pyocyanin was demonstrated. The confocal and SEM imaging served to substantiate this activity.

### Materials and Methods

#### Bacterial strain

*Pseudomonas aeruginosa* isolated from milk using standard protocols and identified using 16S rDNA sequence analysis [13] followed by BLAST (Basic Local Alignment Search Tool) [14] was maintained in nutrient

agar slants at 4°C. The eight food borne pathogens previously isolated from foods, viz. include *Bacillus altitudinis* (KF460551), *Bacillus pumilus* (KF460552), *Brevibacterium casei* (KF573739), *Staphylococcus warneri* (KF573740), *Micrococcus luteus* (KF573741), *Bacillus niacini* (KF573743), *Bacillus sp.* (KF573744) and *Geobacillus stearothermophilus* (KF573747). They were screened for biofilm forming ability, and quantified; their molecular characterization, enzyme profiling and antibiogram construction was also carried out [15].

#### Pyocyanin

Pyocyanin produced by strain *Pseudomonas aeruginosa* (BTRY1), was extracted and characterized using various spectroscopic methods namely UV Visible spectroscopy, Nuclear Magnetic Resonance (NMR) spectroscopy and Fourier Transform Infra-Red spectroscopy (FTIR) [16]. The compound was found to have no hemolytic and cytotoxic activities along with a high free radical scavenging activity [16]. The Biofilm inhibitory concentration (BIC) of the pigment was established by liquid broth assay.

#### Antibiofilm activity and determination of Biofilm inhibitory concentration (BIC)

Extracted pigment was analysed for its ability to control biofilm formation. Microtitre 96 well plates were used for antibiofilm assay [17]. Briefly, 230 µL of tryptone soy broth (TSB) (HiMedia, Mumbai, India) was added to the wells, followed by 20 µL each of the bacterial culture (OD<sub>600</sub> =1), in triplicates for each test organism and incubated aerobically for 24 h at 37°C. 10 µL of

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pyocyanin was added to respective wells and incubated for 24 h at 37°C. Negative control included only TSB. The contents of the plates were poured off; washed thrice with phosphate buffer (0.01 M, pH 7.2) and attached bacteria fixed with methanol. After 15 minutes, plates were decanted, air dried and stained with 1% crystal violet for 5 minutes and excess stain rinsed under running tap water and air dried. The dye bound to adherent cells was extracted with 33% (v/v) glacial acetic acid, and measured at 570 nm using a UV-VIS spectrophotometer (Schimadzu, Japan). Based on absorbance ( $A_{570}$ ) they were graded  $A = A_c =$  No biofilm producers;  $A_c < A =$  Weak biofilm producers;  $2A_c < A =$  Moderate biofilm producers;  $4A_c < A =$  Strong biofilm producers; where cut off absorbance  $A_c$  was the mean absorbance of the negative control. Concentration of pyocyanin used for the assay was estimated, serially diluted and checked for antibiofilm activity. BIC is defined as the minimum concentration which inhibits biofilm formation. All tests were repeated thrice independently and statistically analysed [18, 19]. Finally, the percentage of reduction in biofilm formation was calculated as:

$$\% \text{ in biofilm reduction} = (\text{OD of Control} - \text{OD of Test}) / \text{OD of Control} \times 100$$

Statistical evaluations was done by ANOVA, followed by Sign Test using StatsDirect statistical software (version 3.0, Cheshire, UK) computer program. If there are more positive as negative changes, then  $p > 0.5$  which means the test is significant.

### Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) was used to visualize the antibiofilm effect of pyocyanin on microslides. Culture broth with micro-slide was kept for biofilm formation in the incubator for 24 hours at 37°C, added pyocyanin and incubated under same conditions. Fixation was done with little modifications [20] as shown in the table 1, followed by SEM (JEOL Model JSM - 6390LV).

**Table 1** Fixation steps in Scanning electron microscopy.

Steps	Solution	Time	Repetitions
Primary fixation	2.5% gluteraldehyde	1 h RT*	-
Wash	0.1 M Sodium phosphate buffer (pH 7.3)	5-10 min	3-5
	25% ethanol	5 min	-
	50% ethanol	5 min	-
Dehydration	75% ethanol	5 min	-
	90% ethanol	5 min	-
	100% ethanol	5-10 min	2

RT\* =room temperature, Final fixation done by coating the sample with gold and viewed under microscope

### Confocal Laser Scanning Microscopy (CLSM)

For Confocal Laser Scanning Microscopy, biofilms on micro-slide were fixed using Gluteraldehyde (2.5%), followed by To-pro-3 staining (diluted 1:1000 in PBS) for 10 min in dark at room temperature. They were observed and photographed using confocal imaging system (Leica TCS SP 5) [21]. To-pro-3 stains live cells since it is a nuclear stain and can be seen as red spots due to the far-red fluorescence of the dye. Therefore,

the pixel intensity of the red coloured spots appeared in the images before (Control) and after (Treated) treatment with pyocyanin can be quantified.

The quantification of data for the confocal microscopy was by Image J software (Image J 4.8v/ Java 1.6.0\_20, 64-bit) [22] and the graph plots were generated from the software in the form of Red, Green, Blue (RGB) plots. The variations seen in the RGB plots before and after treatment with pyocyanin are directly related to the pixel intensity provided by the software.

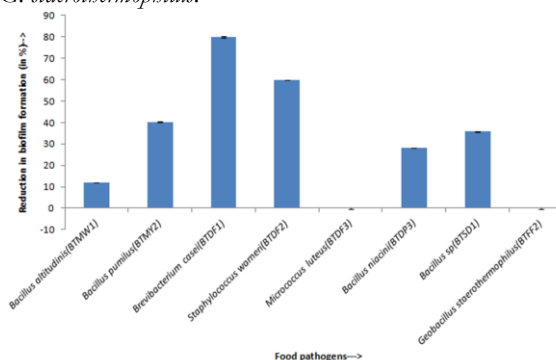
## Results and Discussions

### Antibiofilm activity and Determination of Biofilm Inhibitory concentration (BIC)

The antibiofilm activity was tested with pyocyanin at 1.245  $\mu\text{g}/\text{mL}$  with serial dilutions, and the biofilm inhibitory concentration (BIC) for pyocyanin was calculated to be  $2 \times 10^{-2} \text{ ng}/\mu\text{L}$ . There was no associated cytotoxicity with pyocyanin. The concentrations for cytotoxicity studies ranged from 6.25 -100  $\mu\text{g}/\text{mL}$  along with the Biofilm Inhibitory Concentration at nanogram quantity (Unpublished data). The BIC was found to be much lower compared to the Minimum Inhibitory Concentration (MIC) of pyocyanin calculated for all nine pathogens which was 0.6225  $\mu\text{g}/\text{mL}$  [16].

The study showed that pyocyanin had a profound inhibitory effect on the biofilm forming capability ( $p > 0.5$ ) of six out of the eight isolates tested. It caused significant reduction of biofilm formation by *B. altitudinis*, *B. pumilus*, *B. casei*, *S. warneri*, *B. niacini* and *Bacillus sp.* (Figure 1). However, biofilm formation by *M. luteus* and *Geobacillus stearothermophilus* could not be controlled.

**Figure 1:** Percentage reduction in biofilm formation of pathogens by pyocyanin. The bars indicate percentage of reduction in biofilm formation by each pathogen. 80% reduction in *B. casei*, 60% in *S.aureus*, 40 % in *B. pumilus*, 36% in *Bacillus sp.*, 28 % in *B. niacini*, 12 % in *B. altitudinis* and no reduction in the case of *M. luteus* and *G. stearothermophilus*.



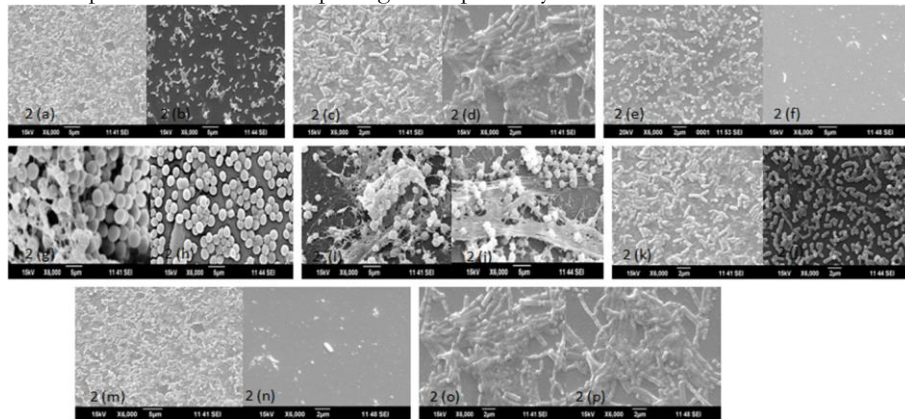
### Scanning Electron Microscopy

The scanning electron microscopy also helped to illustrate the effect of pyocyanin on the biocontrol of biofilms of eight food borne pathogens. The micrograph (Figure 2) shows the difference in the

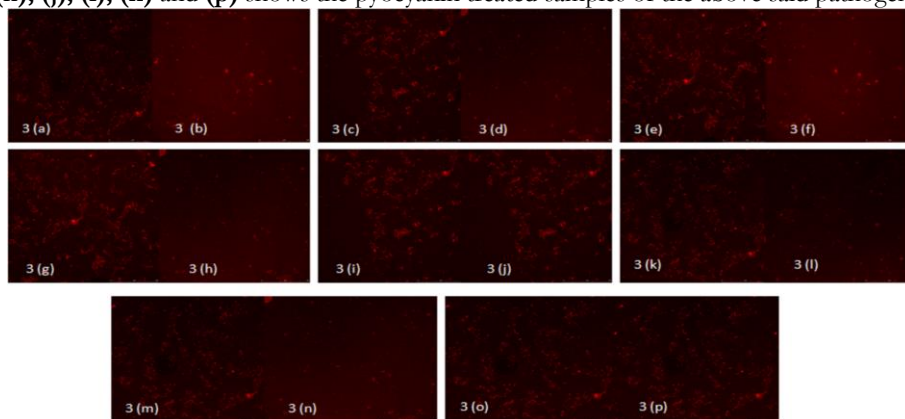
untreated and pyocyanin treated samples of the eight tested pathogens and clearly confirm the reduced microbial presence in the pyocyanin treated slide

compared to control (untreated). Figure 2 shows the Scanning electron micrographs showing the effect of biofilm control by pyocyanin.

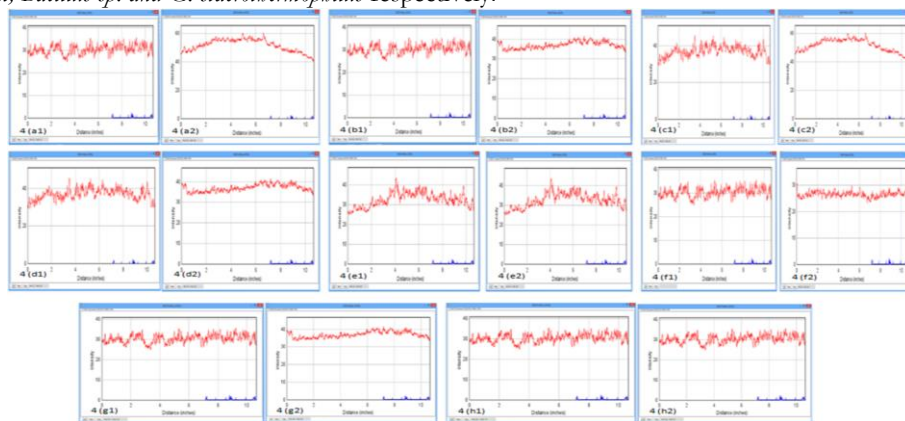
**Figure 2.** Scanning electron micrographs showing the effect of biofilm control by pyocyanin. 2 (a), (c), (e), (g), (i), (k), (m) and (o) shows the control (untreated) samples of *B. altitudinis*, *B. pumilus*, *B. casei*, *S. warneri*, *M. lutues*, *B. niacini*, *Bacillus sp* and *G. staerothermophilus* respectively while 2 (b), (d), (f), (h), (j), (l), (n) and (p) shows the pyocyanin treated samples of the above said pathogens respectively.



**Figure 3.** CLSM images after To-pro 3 staining. 3 (a), (c), (e), (g), (i), (k), (m) and (o) shows the control samples of *B. altitudinis*, *B. pumilus*, *B. casei*, *S. warneri*, *M. lutues*, *B. niacini*, *Bacillus sp* and *G. staerothermophilus* respectively while 3 (b), (d), (f), (h), (j), (l), (n) and (p) shows the pyocyanin treated samples of the above said pathogens respectively.



**Figure 4.** The RGB plots with respect to the corresponding confocal micrographs generated by Image J software. 4 (a1), (b1), (c1), (d1), (e1), (f1), (g1) and (h1) shows the RGB plots of control samples of *B. altitudinis*, *B. pumilus*, *B. casei*, *S. warneri*, *M. lutues*, *B. niacini*, *Bacillus sp*. and *G. staerothermophilus* respectively while 4 (a2), (b2), (c2), (d2), (e2), (f2), (g2) and (h1) shows the RGB plots of pyocyanin treated samples of *B. altitudinis*, *B. pumilus*, *B. casei*, *S. warneri*, *M. lutues*, *B. niacini*, *Bacillus sp*. and *G. staerothermophilus* respectively.



### Confocal microscopy

Similarly, confocal microscopy was also evidence for the biofilm decrease by pyocyanin. The micrographs (Figure 3) clearly show not only a reduction of microorganisms, but also in the biofilm formation by the test (Pyocyanin treated) compared to control (Pyocyanin untreated). Live cells were indicated by To-pro stain. The reduced intensity due to the reduction in the live cells on treatment with pyocyanin was easily visible, which signified the shrinking of biofilm formation. Figure 3 shows CLSM images after To-pro 3 staining.

The intensity data of the confocal images were analyzed using Image J, which produced Red Green Blue (RGB) graphs for each image. Figure 4 represents corresponding RGB plots generated from the software for the eight food borne pathogens. Figure 4 The RGB plots with respect to the corresponding confocal micrographs generated by Image J software.

This study evaluated the antibiofilm activity of pigment produced by *Pseudomonas aeruginosa* (BTRY1) using *in vitro* inhibition assay on microtitre plates, against strong biofilm producers. Among the several pigments of Pseudomonads, pyocyanin is the major antibacterial agent, with the inhibitory effect associated with the 1-hydroxy phenazine component [23]. Pyocyanin production is a widely-accepted criterion for distinguishing *Pseudomonas aeruginosa* from other closely related organisms. Pyocyanin produced by strain BTRY1 showed good antibiofilm activity against the test biofilm producers, normally present in the food industry.

The anti-biofouling activity of a red pigment prodigiosin from *Serratia marcescens* against *Staphylococcus aureus* was previously reported [24]. Similarly, reports are available for antibiofilm activity by melanin [25] against biofilm produced by *Pseudomonas aeruginosa* itself. These reports substantiate the application of pigments against biofilms produced by bacterial pathogens.

The identification of several natural compounds that inhibited biofilm formation by clinical isolates of *Klebsiella pneumoniae* was done by Magesh and coworkers [26], where out of the six compounds that inhibited biofilm formation, reserpine and linoleic acid were potent biofilm inhibitors and their BICs were in milligram quantities. It is very well known that the quorum sensing pathways are inhibited by very low concentrations of natural compounds [27]. Natural compounds like embelin and piperine were required in milligram quantities to control biofilm formation by *Streptococcus mutans* SM06 [28].

The significance of diverse natural products has been perceived by humans because of their gainful properties. Comprehensively, numerous classes of plant auxiliary metabolites have shown their potential as antimicrobials or synergists of different items [29]. Along these lines, phytochemicals are a major source of substance with assorted qualities and are important segments of the current pharmaceuticals [30,31].

BIC of pyocyanin was in ng/ $\mu$ L therefore promising efficient biocontrol of biofilms at low concentrations. The Scanning electron micrographs and Confocal laser scanning micrographs confirmed the biofilm biocontrol capability of pyocyanin.

The study revealed the significance of bioactive compounds as alternatives to the amplified use of antibiotics. The biofilm forming food pathogens in this study showed multiple antibiotic resistance (MAR), when tested against commonly used antibiotics [32].

In recent years, drug resistance of human pathogenic bacteria has been widely reported. In addition, persistent infections were also attributed to enhanced resistance of bacteria in biofilm [33]. This leads to huge economic losses and pressures the medical community to find alternative approaches for treatment of diseases related with biofilms. Consequently, efforts are being applied to discover efficient antimicrobial molecules not amenable to bacterial resistance mechanisms, including those in biofilms [34]. Some natural products have distinctive properties that make them perfect candidates for these highly-required niche- based therapeutics [35].

Thus, pyocyanin proved an effective agent both in control of growth and *in vitro* biofilm formation, and can be considered to as another measure to counter current antibiofilm strategies. This could very well assist in the gradual reduction in multiple resistance of pathogens emerging in the food industry.

### Conclusions

Biofilms are widespread and a bane in the food based industry, with several food related disease outbreaks being associated. The biofilm biocontrol was achieved using a bioactive compound pyocyanin from *Pseudomonas aeruginosa* and was observed to show a great percentage of reduction in the biofilm formation of different foodborne pathogens. SEM and CLSM showed greater variation in the microbial mass and biofilm formation on treatment with the compound. Pyocyanin can thus be considered as an alternate measure to counter current antibiofilm strategies.

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