

Pyocyanin Pigment Extracted from *Pseudomonas Aeruginosa* Isolate as Antimicrobial Agent and Textile Colorant



Science

KEYWORDS : *Pseudomonas aeruginosa*. Pyocyanin. Antimicrobial activity. Phenazine compounds. Textile

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ABSTRACT

Pseudomonas aeruginosa produces an assortment of extracellular pigments, of which phenazines comprise a significant portion. Pyocyanin is the main phenazine pigment associated with this particular organism. The aim of this study was to produce pyocyanin by isolate of *P. aeruginosa*, then tested the antibacterial and antifungal activity of the pyocyanin. It also aimed to investigate the possibility of using this pigment as textile colorant. In this study, L.16 isolate of *Pseudomonas aeruginosa* was obtained from a previous study in Saudi Arabia. The production of pyocyanin by these isolate was investigated. The amount of pyocyanin was determined spectrophotometrically. Pyocyanin was extracted by chloroform, the results provide evidence that the pyocyanin pigment produced by *P. aeruginosa* isolate has antimicrobial activity and can be effectively used as a dye for some textile materials.

Introduction

Pseudomonas aeruginosa is an opportunistic, Gram-negative bacterium and is one of the most commercially and biotechnologically valuable microorganism. Strains of *P. aeruginosa* secrete a variety of extracellular pigments of which phenazines comprise the most significant portion. Phenazine compounds have a variety of biotechnological applications, especially as redox agents and antibiotics. (Jayaseelan *et al.*, 2014).

Many types of soluble pigments are produced by *P. aeruginosa*, including pyocyanin, pyoverdine, pyorubin (red) and pyomelanin (brown). (Krieg and Holt, 2001). The most well studied being pyocyanin which is responsible for the blue-green color characteristic of *Pseudomonas* spp. It is considered both as a virulence factor and a quorum sensing signaling molecule for *P. aeruginosa*. No other species of Gram-negative non-fermenting bacteria produce pyocyanin, making its presence helpful in identifying the organism. (Meyer, 2000).

The most characteristic feature of *Pseudomonas aeruginosa* is the production of soluble pyocyanin pigment: a water-soluble blue-green phenazine compound. From the beginning, pyocyanin had been used as a reversible dye with a redox potential similar to that of menaquinone. Pyocyanin has various pharmacological effects on prokaryotic cells; its biological activity is related to similarity in the chemical structure to isalloxazine, flavoproteins, flavin mononucleotide and flavin adenine dinucleotide compounds. (Ohfuji *et al.*, 2004). The pigment has antibiotic property controlling other microbes and can be used as a biocontrol agent.

To date, some reports (Hernandez *et al.*, 2004) and (Whelan *et al.*, 2006) have documented the colorant production from *Pseudomonas* sp. currently, no studies have been reported on the applications of phenazine colorants in textiles. Moreover, there is an increasing interest in adding value to textiles with the use of natural products. (Saranya *et al.*, 2012).

Materials and Methods

Pyocyanin production

P. aeruginosa isolate was grown on King's B broth medium described by King *et al.* (1954). King's B broth medium contains Protase Peptone 20 g, Dipotassium hydrogen phosphate 1.5 g, Magnesium sulphate. Heptahydrate 1.5 g, Glycerol 10 ml and 990 distilled water and pH 7.2±0.2. Sterilized medium was incubated at 30 °C for 7 days.

Extraction of pyocyanin pigment

P. aeruginosa was grown in broth media (mentioned previously) until pigment was produced. The broth culture was then centrifuged at 4000 rpm for 25 min, finally, sterilization of supernatant was performed using filtration 22 µm Millipore bacterial filter. This filtrate was used for extraction. The procedure of extraction of pigment described by Ingledew and Campbell, (1969) and (Saha *et al.*, 2008), with some modification. The pigment was extracted from supernatant with chloroform at a ratio of 1: 2 (v/v) for 2 hrs. The blue chloroform was washed with acidified water (0.2M HCl), under continuous stirring until complete extraction of whole pyocyanin, such treatment converted the blue pigment to acidic form (Red). The acidified layer (the red layer) was collected and the pH was neutralized to 7 by 0.4 M borate-NaOH buffer (blue color). The extraction was repeated 3 times until complete extraction and the purity of the pigment. The absorbance of this solution was measured at 520 nm. Concentrations expressed as micrograms of pyocyanin produced per milliliter of culture supernatant. (Es-sar *et al.*, 1990).

Concentration of pyocyanin (µg/ml) = O.D.₅₂₀ × 17.072

The chloroform was evaporated to dryness in an oven at 45°C and the formed needle-like crystals were kept as a powder in the refrigerator.

Pyocyanin as antibacterial

Antibacterial assay was carried out by well diffusion technique for pyocyanin pigment along with DMSO was taken as control. Wells with 0.5 mm diameter were made on sterile Nutrient agar plates. Four tested bacteria, namely *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* were added to culture medium, and 0.1 ml of these cultures was inoculated into each Petri dishes and different concentration of pyocyanin pigments (5, 10, 15, 20 and 25 mg/ml) were added to the wells. Plates were incubated at 35°C for 24 h. The antibacterial activity of the pigment was determined by measuring the growth inhibition around the well. (Saha *et al.*, 2008).

Pyocyanin as antifungal

Antifungal activity of pyocyanin was detected against *Aspergillus niger* and *Rhizoctonia solani*. Sabouraud's dextrose agar (SDA) was used for fungal cultures. A total of 5 mm diameter wells were punched into the agar and filled with 100 µL of pyocyanin for each concentration (5, 10, 15, 20 and 25 mg/ml) and incubated at 28 °C. 100 µL of dimethyl sulfoxide was taken as control. After 5 days of incubation

tion, the antifungal activity of the pigment was determined by measuring the growth inhibition around the well. All experiments were carried out in triplicate.

The antifungal activity of pyocyanin against *C. albicans* was determined using an agar diffusion method. The standard solution of testing strain was swabbed over SDA plates. After 10 minutes, various concentrations (5,10,15,20 and 25mg/ml) were placed on different points of the agar surface. All plates were incubated at 30°C for 24 to 48 hours, and the inhibition zone was evaluated.

Pyocyanin as textile colorant

In the present study, the scope for probable application of the bacterial pigment was evaluated for different grades of textile materials commercially available in the market, which included Cotton and Linen fabric. Each material was cut into equal size of 20 cm². The bacterial pigment was applied to the cloth material and was allowed to dry at room temperature. A sample of each type of cloth was ironed for 20 minutes. Another sample of Cotton and Linen Fabric has not been ironed. The material was subjected to soaping in order to remove the unfixed dye substances present at the surface level and dried at room temperature (28 ± 2°C). In all the experiments, white cloth material was taken as a control. (Poorniammal *et al.*, 2013)

Results and discussions

Extraction of pyocyanin pigment

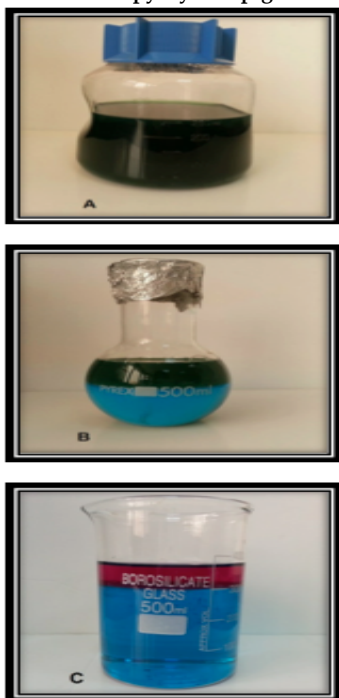


Fig. (1):

A)- Supernatant of culture

B)- Extraction of supernatant using chloroform (1:2 v/v)

C)- Addition of 20% of 0.2 N HCl

The pigment was extracted using chloroform which produced a blue color compound which was further confirmed by adding 0.2N HCl. A change in color to red indicated only the presence of pyocyanin pigment which was used for further study Fig (1).

Antibacterial activity of Pyocyanin

Table (1): Antibacterial activity of pyocyanin

Concentrations of pyocyanin mg/ml	<i>B. subtilis</i> ATCC 6633	<i>S. aureus</i> ATCC 25923	<i>E. coli</i> ATCC 25422	<i>K. pneumonia</i> ATCC 700603
	Inhibition Zone in mm Mean ± SD			
5	27.66 ± 1.04	10.33 ± 0.58	23.33 ± 0.58	15.67 ± 1.2
10	29 ± 1.0	14.67 ± 1	27 ± 1.7	20 ± 0.0
15	31 ± 2.0	19.67 ± 1.3	29.33 ± 2.08	27.33 ± 0.58
20	34 ± 0.0	25 ± 0.0	35 ± 0	30.67 ± 1.15
25	36 ± 1	26.33 ± 1.2	37.76 ± 1.15	32.33 ± 1.2

Data in Table1 showed that the antibacterial activity of pyocyanin against tested bacteria (*B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25422 and *K. pneumonia* ATCC 700603) increased by increasing of the pyocyanin concentrations (5, 10, 15, 20, 25 mg/ml). The inhibition zones were different which depended on the isolates. The inhibition zone of *B. subtilis* ATCC 6633 ranged from 27.67±1.04 to 36±1 mm but inhibition zones of *S. aureus* ATCC 25923 ranged from 10.5±0.58 to 26.3± 1.2 mm. The antibacterial activity of pyocyanin against *E. coli* ATCC 25422 showed inhibition zone increased by increasing in pyocyanin concentrations, it ranged from 23.33±0.58 to 37.67±1.15. The inhibition zones of *K. pneumonia* ATCC 700603 ranged from 15.67±1.2 to 32.33±1.2 mm. Pyocyanin pigment of *P. aeruginosa* showed a greater effect against all Gram positive and Gram negative tested bacteria, and the antagonistic activity against tested bacteria increased by increasing of the pyocyanin pigment concentration. (Saha *et al.*, 2008) reported that pyocyanin has antagonistic activity against pathogenic bacteria like *Salmonella paratyphi*, *E. coli* and *Klebsiella pneumonia*. Our results do not agree with El-Shouny *et al.*, (2011) who found that the Gram-positive bacteria were more susceptible to the antibiotic action of pyocyanin than were the Gram negative bacteria. These results are in agreement with the results of Makarand *et al.*, (2007) who recorded an antimicrobial activity of the pyocyanin against strains of *Bacillus subtilis*, *Candida albicans*, and *Escherichia coli*. However, others demonstrated that activity of phenazine antibiotics are concentration dependence, therefore; when the concentration of phenazine increased, the biological activity would be increased. (Stephen and John, 1981).

Antifungal activity of pyocyanin

Table (2): Antifungal activity of pyocyanin

Concentrations of pyocyanin mg/ml	<i>B. subtilis</i> ATCC 6633	<i>S. aureus</i> ATCC 25923	<i>E. coli</i> ATCC 25422	<i>K. pneumonia</i> ATCC 700603
	Inhibition Zone in mm Mean ± SD			
5	27.66 ± 1.04	10.33 ± 0.58	23.33 ± 0.58	15.67 ± 1.2
10	29 ± 1.0	14.67 ± 1	27 ± 1.7	20 ± 0.0
15	31 ± 2.0	19.67 ± 1.3	29.33 ± 2.08	27.33 ± 0.58
20	34 ± 0.0	25 ± 0.0	35 ± 0	30.67 ± 1.15
25	36 ± 1	26.33 ± 1.2	37.76 ± 1.15	32.33 ± 1.2

This study showed the antagonistic activity of pyocyanin Produced by *P. aeruginosa* against *R. solani*, *A. niger* and *C.albicans*. Data in Table2 showed that the antagonistic activity against tested fungi increased by increasing of the pyocanin pigment concentration (5, 10, 15, 20, 25 mg/ml). Data in Table 2 shows that the inhibition zone were different which depended on the tested fungi. There is no inhibition zone at conc. 5mg/ml for both *R. solani* and *A. niger*. The antagonistic activity of pyocyanin against *R. solani* ranged from 13.67 ± 1.15 to 24 ± 1.0 mm at conc. 10 to 25 mg/ml, and the inhibition zone ranged fome 15.33 ± 1.25 to 21.33 ± 1.53 mm against *A. niger* at conc. 15 to 25 mg/ml. Finally the antagonistic activity of pyocyanin against *C.albicans* ranged from 13.67 ± 0.58 to 25.67 ± 1.5 mm at conc. 5 to 25mg/ml.

Antifungal activity of the Pyocyanin produced by *P. aeruginosa* was subjected to *R. solani*, *A. niger* and yeast as *C. albicans*. Results showed that the inhibition zone were different which depended on the concentrations of pigment. There is no inhibition zone at low concentration of pyocyanin for both *R. solani* and *A. niger* but inhibited at higher concentrations. Kerr *et al.*, (1999) explained the antifungal activity of pyocyanin and the strong antagonism against *Candida albicans* and *Aspergillus fumigatus*. Bakthavatchalu *et al.*, (2013) reported that pyocyanin produced by *P. aeruginosa* play an important role as an indicator of phytopathogens pathogens like *R. solani*. The pyocyanin extract of *P. aeruginosa* was isolated from rhizosphere soil can be used as biosupplement antagonism against fungal rice pathogens such as *Helminthosporium oryzae*, *Pyricularia oryzae*, *Rhizoctonia solani*. (Jayaseelan *et al.*, 2014). The antibacterial and antifungal nature of the pigment proves attractive for the topical treatment of wound infections. (El-Shouny *et al.*, 2011). The antagonistic effects of almost all of phenazine characteristic redox activity is thought to kill off competing fungi through the production of reactive oxygen species. (Chin-A-Woeng *et al.*, 2003).

4- Pyocyanin as textile colorant

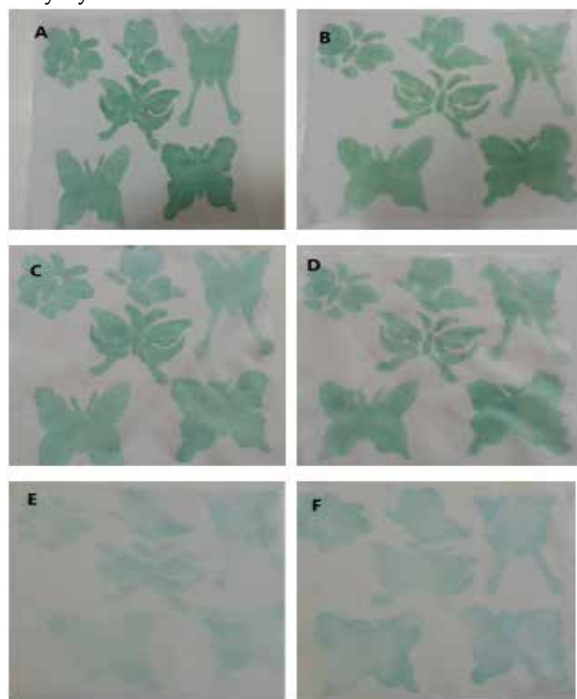


Fig. (3): Pyocyanin as textile colorants

A): Pyocyanin pigment on cotton fabric was fixed by heat iron.

B): Pyocyanin pigment on linen fabric was fixed by heat iron.

C): Pyocyanin pigment on cotton fabric was fixed by heat iron and washed as described previously.

D): Pyocyanin pigment on linen fabric was fixed by heat iron and washed as described previously.

E): Pyocyanin pigment on cotton fabric was washed without fixed by heat iron.

F): Pyocyanin pigment on linen fabric was washed without fixed by heat iron.

In this study the fabrics were used are cotton and linen. Results presented in Fig (3) clearly evidence that the pyocyanin pigment produced by *P.aeruginosa* L.16 can be effectively used to dye all the textile materials studied. During the wash performance studies with the textile (E, F) materials treated with pigment, it was found that the pigment is lost from the cloth untreated with heat, after washing in soap solution at room temperature ($28 \pm 2^\circ\text{C}$). Whereas, the loss of pigment from the same textile materials treated with heat was found to be less. So it is inferred that use heat is an effective for treating the dyed textile materials. During the wash performance studies with the textile (E, F) materials treated with pigment, it was found that the pigment is lost from the cloth. Untreated heat, after washing in soap solution at room temperature; $28 \pm 2^\circ\text{C}$. Whereas, the loss of pigment from the same textile materials treated with heat was found to be less. So it is inferred that we can use the pyocyanin pigment as textile colorants, that's after the treatment of fabric with heat.

Phenazines, namely pyorubin and oxychlororaphin, were extracted from *Pseudomonas* sp., purified and the dyeing potential of the phenazines for silk dyeing was examined. (Saranya *et al.*, 2012). Technically speaking, biosynthesized pigments can serve as major chromophores for further chemical modifications, which could lead to colorants with a broad spectrum of colors. (Hobson and Wales, 1998). Besides, some natural colorants, especially anthraquinone type compounds, have shown remarkable antibacterial activity in addition to providing bright colors. Frandsen *et al.*, (2006) which could serve as functional dyes in producing colored antimicrobial textiles. Alihossein *et al.*, (2008) characterized the bright red pigment prodigiosin from *Vibrio* spp. and suggested that it could be used to dye many fibers including wool, nylon, acrylics, and silk.

Conclusions

Pseudomonas aeruginosa has been found in all sources of environment and recognized by its production of the pyocyanin pigment. Pyocyanin, the characteristic blue pigment of *P. aeruginosa*, is a redox-active phenazine compound which contributes to the virulence of *P. aeruginosa* as an opportunistic pathogen and has antibiotic activity against a range of bacteria and fungi. Pyocyanin is a natural product which has the ability to act as a bio-control agent, thus helping to create an eco-friendly solution for the replacement of chemical pesticides. Pyocyanin can be processed technically by using various parameters such as an appropriate media for pigment production from various environmental sources. The primary screening of this metabolite is done by chloroform extracts from the media.

The results of the present study clearly indicated that the pyocyanin pigments of *P. aeruginosa* can be produced in a laboratory. The purified pigment has bioactive properties and is active against bacterial and fungal pathogens. It can be concluded that pyocyanin possesses antimicrobial activity against pathogens and may be used topically in susceptible cases. Also, this study recommends to using pyocyanin in wider industrial fields as a natural colorant of materials used in the manufacture of fabrics and carpets, paper, and the possibility of using inks industry.

ACKNOWLEDGMENT

I express my gratitude to **Allah** for providing me the blessings. I would like to express my deepest gratitude to my supervisor **Prof. Salha Alzahrani** who provided wonderful guidance, advice and continuous encouragement during my studies. Thanks to Faculty of Science, Biological Department of Sulaimaniya and Faisaliah branches, **King Abdulaziz University** for their cooperation and guidance.

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