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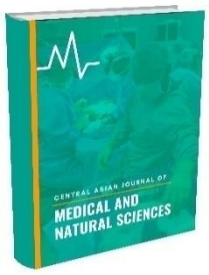

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Comparative Analysis of Pyocyanin Production and Antibacterial Efficacy on Diverse Culture

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Abstract: We investigated the impact of culture medium on pyocyanin production by Pseudomonas aeruginosa. Our study included variations in media, temperatures, and durations, while also assessing the antibacterial activity of pyocyanin against pathogens. Our aim was to understand the factors influencing pyocyanin production and its therapeutic potential

For this study, we obtained 26 strains of Pseudomonas aeruginosa from various sources. Out of these, 17 strains had the ability to produce pigment. We selected the strain that demonstrated the highest production of pigment for all subsequent experiments.

To investigate the influence of culture media on pyocyanin, we cultured the selected strain on various media types, including nutrient agar, Brain Heart Infusion agar, tryptic soy agar, CT agar, Muller Hinton, Nutrient broth, Peptone water, and Tryptic Soy broth. The incubation conditions were varied by testing at both 37°C and 35°C and at different time intervals (24, 48, 72, and 96 hours), as well as at different pH levels (7.5 and 7.8). Following culture growth, we extracted the pyocyanin pigment and examined its antibacterial efficacy.

This study investigated the production of pyocyanin by P. aeruginosa and found that it varies depending on the culture medium used. Pigment concentration was measured using a spectrophotometer at 520 nm. The highest mean optical density was observed in Tryptic soy agar (0.364), followed by Tryptic soy broth (0.322) and the lowest mean optical density was observed in Peptone water (0.08). The optimal condition pyocyanin production were found to be a temperature of 35°C, pH of 7.8 and there is no statistically significant difference in the production of pyocyanin between the two incubation times of 72 and 96 s. The study also

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evaluated the antibacterial activity of pyocyanin and found that the pigment produced on the agar had a high ability to inhibit the growth of pathogenic bacteria and the swarming of Proteus mirabilis. However, the pigment exhibited varying degrees of antibacterial activity against pathogenic bacteria.

Key words: Pyocyanin production, antibacterial efficacy, Pseudomonas aeruginosa, pathogens, pigment, incubation, tryptic soy.

Introduction

Pseudomonas aeruginosa is known for its versatility and ability to thrive in various environments. It can be found in a wide range of habitats, including clinical samples, water, soil, plants, animals, and even man-made environments like hospitals. This adaptability is due to its ability to produce a variety of extracellular products and enzymes that allow it to survive and compete in different environments. In clinical settings, P. aeruginosa is a common cause of opportunistic infections, particularly in immunocompromised individuals. It is also a major concern in hospital settings due to its ability to form biofilms on medical devices, making it resistant to antibiotics and disinfectants. (1).

P. aeruginosa is an opportunistic pathogen responsible for nosocomial infections and a wide range of diseases, including pulmonary disease, cystic fibrosis, and wound infections. Infections caused by this bacterium can be life-threatening and potentially fatal. (2)

90% of Pseudomonas aeruginosa strains were produced pyocyanin (3). Pyocyanin has a detrimental effect in cystic fibrosis disease, which enables P. aeruginosa to persist in the lungs of cystic fibrosis patients. It can be isolated from the sputum of cystic fibrosis patients. (4) Pyocyanin is blue green pigment soluble in water, synthesis in active culture of P. aeruginosa, Pyocyanin has antimicrobial effect against different microorganisms

In addition to its role in cystic fibrosis disease, pyocyanin pigment is also an important virulence factor and quorum sensing (QS) signaling molecule for P. aeruginosa. Despite the fact that Pseudomonads are often classified as pathogenic bacteria, the ability of this bacterium to produce antimicrobial pigment makes it a potential agent for biological control. (7)

Scientific research has demonstrated the antibacterial activity of pyocyanin.(5) Numerous studies have confirmed the broad-spectrum antimicrobial activity of pyocyanin against fungal cells, bacteria, and eukaryotic cells. Furthermore, it has been found to possess anti-tumor, anti-malaria, and anti-parasitic properties (6)

Several studies have investigated pyocyanin production by P. aeruginosa in various sample types, including clinical, environmental, and industrial sources. Some studies have reported that P. aeruginosa strains isolated from clinical sources, such as burn wounds, produce higher levels of pyocyanin compared to strains isolated from environmental sources, such as soil or water. However, other studies have reported contrasting results, with some environmental strains producing higher levels of pyocyanin than clinical strains.

In a study conducted by G. Young in 1947, the relationship between the culture medium type and the amount of pyocyanin produced was investigated. Additionally, a previous study has demonstrated that the addition of certain nutritional supplements can stimulate pyocyanin production. (9 DeBritto et al 2020)

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The objective of this study was to identify the optimal culture medium for pyocyanin production. The comparison was made using optical density measurements. Initially, the effect of different media on pyocyanin production was determined, followed by an investigation of the antibacterial properties of the pigment against pathogenic bacteria.

Material and method

Collection of samples

The study isolated a total of 47 Pseudomonas aeruginosa isolates from 100 samples obtained from various sources. Among the sources were 30 samples of water from dental units, 15 wound swabs, 15 urine samples, 15 soil samples, 10 sputum samples, and 15 burn infections, as shown in Table 1.

Isolation of *P. aeruginosa*

All samples were cultured on MacConKey agar and incubated for 24 hrs. at 37 oC. Colony shape, color and Oder were used in diagnosis of P.aeruginosa. The strains that give positive oxides test were examined for production of pyocyanin on cetrimide agar.

Examination of pyocyanin production on different culture media

The P. aeruginosa strain with the highest pyocyanin concentration was selected and cultured on various culture media, including N. agar, Tryptic soy agar, Brain heart infusion agar, CT agar, Muller Hinton, N. broth, peptone water, and tryptic soy broth, and incubated at 35°C and 37°C. Pigment production was evaluated every 24 hours, and after four days of incubation, the pigment was extracted from both agar and broth media using chloroform

Pyocyanin Extraction from agar media

After an incubation period of 96 hours, the bacterial growth was harvested from the culture medium by a cover slide. The surface of culture media was washed with distilled water, and the agar was cut into 1cm pieces. To extract pyocyanin, 20ml of chloroform was added and the solution was shaken vigorously until a blue color appeared. This process was repeated three times. The blue color solution was then filtered using a 0.45mm filter paper. Next, 20ml of 0.2mHCl was added to the red color solution, which was collected and measured for optical density at 520nm table 2

A comparison was made using a spectrophotometer to determine the differences in pyocyanin production on different culture media (12)

Pyocyanin Extraction from broth culture

After 96 hours of incubation, the broth culture was subjected to centrifugation at 12,000 × g for 20 minutes at 4°C. The resulting supernatant was collected and mixed with chloroform at a ratio of 1:1.5 (supernatant: chloroform). The mixture was vigorously shaken for 15 seconds until a color change from green to blue was observed. Then, 0.2N HCl was added to the mixture at a ratio of 1:2 (supernatant: chloroform), followed by vigorous shaking and centrifugation at 3000 rpm for 10 minutes. The resulting supernatant containing crude pyocyanin was stored at 4°C

To determine the concentration of pyocyanin in the supernatant, its optical density was measured at 520nm (10).

Antibacterial Activity of pyocyanin

Method 1

To investigate the antibacterial properties of the pigment produced by Pseudomonas aeruginosa, a Tryptic Soy Agar (TSA) medium was used. The bacterial culture was grown on the TSA at 37°C for 72 hours, and the pigment production was confirmed

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The bacterial culture was harvested using a cover slide, and the TSA plate was immersed in chloroform and left to dry. Then, 0.1 µl of a 10^6 dilution of the test bacteria, as mentioned in the table below, was placed on the TSA plate and incubated for 24 hours. The growth of the test bacteria was examined to evaluate the potential antibacterial effects of the pigment.

Method2

Various pathogenic bacteria were cultured on brain-heart broth for 24 hours. The bacterial culture was then spread on a Muller-Hinton agar using a spreader and left to dry for 10 minutes. Wells were made using a cork borer, and each well was filled with 0.1µl of extracted pyocyanin. The plate was then incubated for 24 hours, and the results were observed and recorded in Table 4, figure 1.

Swarming inhibition examination

Tryptic soy agar containing the pyocyanin was inoculated with *proteus mirabilis* and swarming phenomenon was observed after 24 hr incubation at 37

The results

Collection of samples.

Type of sample	No. of sample	No. of p. aeruginosa strain	Produced pyocyanin	% of pyocyanin production
dental units water	30	12	4	33.3%
wounds swab	15	5	2	40%
Urine	15	2	0	0%
Soil	15	5	3	60%
Sputum	10	3	1	33.3%
Burn	15	9	7	77.7%
Total	100	26	17	%65.4

Table 1- The percentage of pyocyanin production according to the types of samples

No.	**Type of culture media**	**O.D/after 72 hrs.**	**OD/after 96 hrs.**
.1	**Tryptic soy agar**	**0.364**	**0.369**
.2	**Tryptic soy broth**	**0.322**	**0.334**
.3	**Nutrient agar**	**0.145**	**0.147**
.4	**CT**	**0.118**	**0.120**
.5	**Muller Hinton**	**0.135**	**0.144**
.6	**peptone water**	**0.080**	**0.082**
.7	**Nutrient broth**	**0.104**	**0.110**

Table 2- The Optical density at 520 nm of pyocyanin on different type of culture media after 72and 96hrs incubation time pH 7.8.

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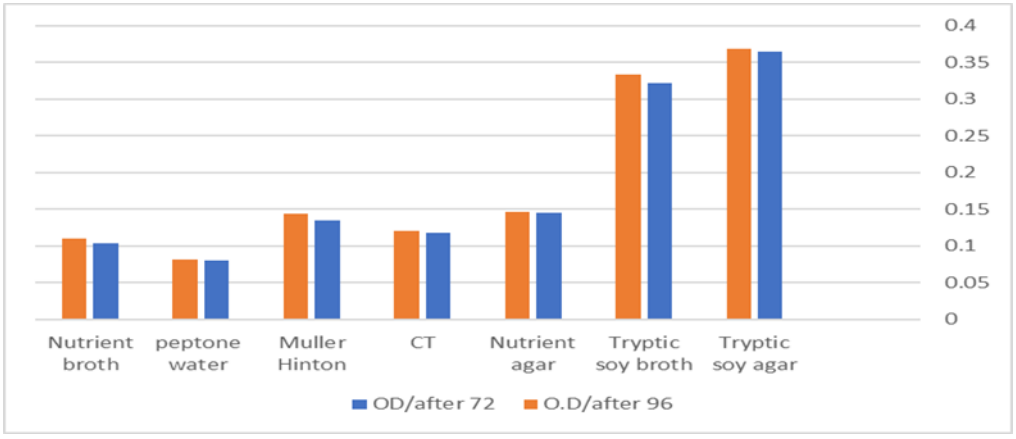

Figure 2:- The optical density of pyocyanin at 520 nm was measured for different types of culture media after 72 and 96 hours of incubation at a pH of 7.8

The highest mean optical density was observed in Tryptic soy agar (0.364), followed by Tryptic soy broth (0.322) and Nutrient agar (0.145). The lowest mean optical density was observed in Peptone water (0.080)

When we used to perform the one-way ANOVA test

The p-value is less than the alpha level of 0.05 Therefore, there is a significant difference in the growth rate of the culture among the different types of culture media

Performing a paired t-test on the data provided in table 2, the results are:

t = 1.0137, df = 6, p-value = 0.132

A two-sample t-test was conducted on the given data, which resulted in a calculated p-value of 0.132. Since this p-value is greater than the commonly used significance level of 0.05 Therefore, we can conclude that there is no statistically significant difference in the production of pyocyanin between the two incubation times of 72 and 96 hours.

No.	Type of bacteria	Method1
.1	P.aeruginosa (not produce pigment) 3 strain	No growth
.2	P.aeruginosa (produce pigment)	No growth
.3	P.aeruginosa (produce pigment)	No growth
.4	P.aeruginosa (produce pigment)	No growth
.5	E.coli	No growth
.6	K. pneumonia	No growth
.7	Proteus mirabilia	No growth
.8	Streptococcus pneumonia	No growth
.9	S.aeures	No growth

Table 3:-antibacterial effect of pyocyanin on different type of pathogenic bacteria method1 .

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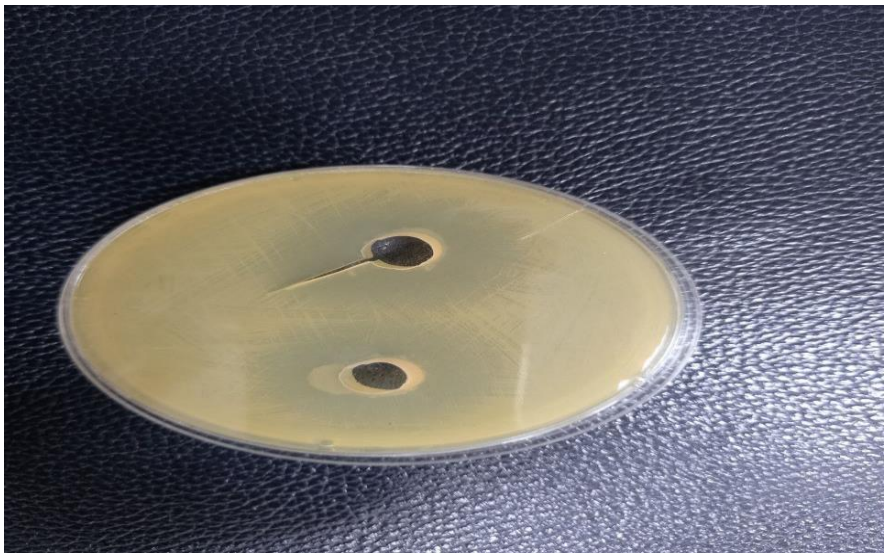

Fig 1: - Antibacterial effect of crude pyocyanin on K. pneumonia cultured on Muller-Hinton agar method2

No.	Type of bacteria	Diameter of inhibition zone /mm
.1	P.aeruginosa (not produce pigment)P1	25
.2	P.aeruginosa (produce pigment)P3	20
.3	P.aeruginosa (produce pigment)P6	0
.4	P.aeruginosa (produce pigment)P7	0
.5	E.coli	22
.6	K. pneumonia	25
.7	Proteus mirabilia	20
.8	Streptococcus pneumonia	14
.9	S.aeures	17

Table 4: - Antibacterial effect of pyocyanin on different type of pathogenic bacteria method2 .

Discussion

Pyocyanin is a blue-green pigment produced by P. aeruginosa, and its production is often associated with virulence and pathogenesis.

The results indicate that the production of pyocyanin varies among different sources. Among the samples tested, the highest pyocyanin production was observed in burn wounds (77.7%), followed by soil (60%), wounds swab (40%), dental units water (33.3%), sputum (33.3%), and urine (0%) table 1.

Interestingly, no pyocyanin production was observed in the urine samples tested, which may suggest that P. aeruginosa strains found in urine are less virulent than those found in other sources. The results also show that the total percentage of pyocyanin production is 65.4%, indicating that P. aeruginosa strains have the potential to produce pyocyanin in a majority of the tested samples.

The study shows that there is a slight increase in the OD values at 96 hours as compared to the values at 72 hours for most of the culture media. Therefore, we can conclude that there is no statistically significant difference in the production of pyocyanin between the two incubation times of 72 and 96 hours

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Among the culture media tested, Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) showed the highest OD values, indicating that they support better bacterial growth. This is consistent with the fact that TSA and TSB are commonly used for culturing a wide range of bacteria due to their nutrient-rich composition.

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