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Screening for Blue-green Pigment Production by *Pseudomonas* Isolates

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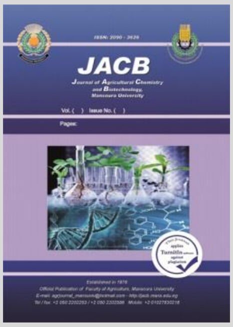

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ABSTRACT

Eight isolates belonging to the genus *Pseudomonas* were isolated from various environmental sources, including soil, water, and food material, and tested for blue-green pigment production. All selected isolates exhibited good production of the blue-green pigment, with three isolates showing high levels of pigment production, yielding 2.13, 3.47, and 5.74 µg/mL when cultured in nutrient broth medium supplemented with 1% waste oil. In an attempt to select an appropriate medium for blue-green pigment production, six different media were tested with all isolates. The Obtained data indicate a significant variation in microbial pigment production among the different culture media. The data revealed that King's medium was the most effective for pigment production compared to nutrient broth and Bushnell Haas media with all tested *Pseudomonas* isolates. On the other hand, the results demonstrated that the supplementation of nutrient broth and Bushnell Haas media enhanced pigment production. Nutrient broth (Oxoid) showed high productivity with all isolates when supplemented with waste oil or glycerol. Maximum pigment production was achieved by isolate W4, yielding 4.98 µg/mL when cultured in waste oil-supplemented nutrient broth.

Keywords: Blue-green pigment, Waste-oil, *Pseudomonas*

INTRODUCTION

Pigments are chromophores that have been widely utilized due to their vibrant colors, which they impart to various substances. Biopigments, or biocolors, are naturally occurring pigments extracted from sources such as plants, animals, and minerals, and have been utilized since ancient times. With the revolution of industry, synthetic dyes gained rapid popularity due to their high productivity, high intensity and high stability and low cost of production (Padhan *et al.,* 2021). Consequently, natural pigments were gradually replaced by synthetic dyes, which have been used in a wide range of applications, including food, agriculture, textiles, electronics and cosmetics (Azman *et al.,* 2018; Huang *et al.,* 2024). Although their commercial advantages, synthetic dyes contribute to environmental pollution because of their non-degradable, toxic and carcinogenic effects on human health (Tkaczyk *et al.,* 2020). Moreover, removing these substances from the contaminated environments requires a complex addressing process and high cost (Mehdizadeh *et al.,* 2020; Orooji *et al.,* 2020).

Increasing awareness of consumers and their interest in safe, eco-friendly products have caused a shift toward naturally occurring pigments. Natural pigments were increasingly used as a sustainable alternative to synthetic dyes due to their biodegradable, non-toxic, and non-carcinogenic properties (Cristea and Vilarem, 2006). Microorganisms, which are considered one of such pigments, have garnered special attention due to their ability to overcome a number of drawbacks associated with pigments obtained from plants or animals. Among microorganisms, bacteria are preferred because of their advantages including short life cycles, independence from weather condition, production of diverse pigments, and ease of large-scale fermentation and downstream processing (Sutthiwong *et al.,* 2014; Celedón and Díaz, 2021; Finger *et al.,* 2019, Pankaj and kumar, 2016). Furthermore, many bacterial strains are capable of utilizing waste materials that are environmental pollutants to secrete bacterial pigments, which made this process more sustainable (Joshi *et al.,* 2003; Kamla *et al.,* 2012). This not only enhances pigment production but also contributes to sustainable waste management, aligning with the principles of a circular economy (Banu et *al.,* 2021; Muscat *et al.,* 2021; Yaashikaa *et al.,* 2022, Grewal *et al.,* 2022).

Bacterial pigments are secondary metabolites distinguished by their various colors, unique molecular structures, and relatively low molecular weights (Grewal *et al.,* 2022). They play significant roles in biological and ecological functions, including photosynthesis (Nowicka & Kruk, 2016), protection against ultraviolet radiation (Moeller *et al.,* 2005), iron acquisition (Cornelis & Dingemans,2013), signaling pathways linked to quorum sensing (Liu & Nizet, 2009) and ecological interactions with other organisms (Viana *et al.,* 2017). Among these pigments, phenazines represent a unique class of nitrogen-containing heterocyclic compounds with diverse physicochemical properties (Pierson & Pierson, 2010). More than 50 naturally occurring phenazines have been identified, with at least 10 types often coexisting in a single organism. Among them, pyocyanin a blue-green pigment produced primarily by *Pseudomonas aeruginosa* is considered the most significant due to its multifaceted biological activities and broad biotechnological potential (Shanmugaiah *et al.,* 2010).

The aim of the present study is to isolate highly effective blue-green pigment producing bacteria and study their productivity on different media.

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MATERIALS AND METHODS

isolation and Screening of pigment producing microorganisms

Various samples of soil, water, and minced meat collected from different locations in Dakahlia Governorate, Egypt, during a screening study for blue-green pigment-producing bacteria using nutrient agar medium (Oxoid, UK). Pigmented colonies were selected and streaked onto nutrient agar plates for purification. Pigmented colonies with a grape-like odor and shiny appearance were subcultured on blood and MacConkey agar (Oxoid, UK) and incubated at 37°C for 24 h. Colonies showing β-hemolysis and non-lactose fermentation were selected as potential Pseudomonas isolates (Vandepitte *et al*., 2003). Standard biochemical tests, including catalase, oxidase, and gelatin liquefaction, as well as the ability to grow at 42°C were performed to confirm identification, based on the methods described by Palleroni (1992). Also, Morphological characteristics such as Gram staining, motility, shape and pigment production were performed as described by (Holt *et al*. 1994).

Selective Isolation of blue-green Pigment Producing bacteria

The different *Pseudomonas* isolates were cultured in 250 ml conical flasks containing 50 ml of nutrient broth supplemented with waste oil. The cultures were incubated in a shaker at 150 rpm and 32 °C for 48 h. Blue-green pigment production was estimated at the end of the incubation period. For comparison, six different synthetic media were evaluated for their effect on pigment production by the selected Pseudomonas isolates. These media included: (1) nutrient broth, (2) nutrient broth supplemented with 1% waste oil, (3) nutrient broth supplemented with 1% glycerol, (4) King's A medium (King *et al*., 1954), (5) Bushnell-Haas medium (Bushnell and Haas, 1941), and (6) Bushnell-Haas medium supplemented with 5 g/L peptone.

Extraction and estimation of blue-green pigment

The extraction protocol was followed as described previously by (Saha *et al.,* 2008). After incubation, the production medium was centrifuged for 20 min at 5000 rpm, and then the cell pellet was discarded while the cell-free supernatant was extracted directly. In a separating funnel, 3 ml of the supernatant was combined thoroughly with 6 ml of chloroform. After vigorously shaking, the chloroform layer was separated from the aqueous solution and its color changed to blue. The concentration of the blue pigment was estimated by measuring the absorbance of acidic solution at 520 nm. Pigment concentration was calculated by micrograms per milliliter according to the following equation (Essar *et al.,* 1990):

Concentration of Pigment (µg/ml) = OD $_{520nm}$ x 17.072

Statistical analysis:

All experiments were conducted in triplicate, and the data are presented as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA (Duncan's multiple range test) with CoStat software version 6.303 to determine significant differences between means.

RESULTS AND DISCUSSION

Data in Table No. 1 show the number of bacterial isolates obtained from soil, water, and food samples. The presented data also indicates the number of isolates presumptively identified as *Pseudomonas* based on their morphological characteristics and pigment production. The data show that one isolate was isolated from agricultural wastewater. The data presented in the table (1) show that three bacterial isolates were isolated from agricultural wastewater in Dakahlia Governorate, one of which was expected to be *Pseudomonas*. Two bacterial isolates were isolated from benzene-contaminated soil, one of which was expected to be *Pseudomonas*. From Nile River water, 12 isolates were isolated, three of which were suspected to be *Pseudomonas*. Three isolates were obtained from irrigation water, one of which was expected to be *Pseudomonas*. Finally, three isolates were isolated from minced meat, two of which were expected to be *Pseudomonas*.

The isolation results can be summarized as 8 isolates were obtained, which are expected to belong to the genus *Pseudomonas*, and which were selected based on the characteristic of the bacterial colonies in the culture medium and on the color of the blue-green pigment produced by the colonies.

Table 1. Classification of isolated bacteria from different samples:

Source of isolation	Number of pigmented bacterial isolates	Number of expected Pseudomonas sp. isolates
Agricultural drainage water from Dakahlia governorate	3	1
Soil polluted by gasoline from Dakahlia governorate	2	1
Nile river water from Dakahlia governorate	12	3
Irrigation water from Dakahlia governorate	3	1
Minced meat sample	3	2

The eight bacterial isolates which are expected to be *Pseudomonas* were tested for their ability of the production of the blue-green pigment by growing them in nutrient broth medium for 48 h. at 30°C with shaking at 160 rpm. The results shown in Table 2 show that all isolated bacteria can produce blue green pigment on nutrient broth medium. Also, data show that there was a clear variation in the ability of the isolates to produce the blue-green pigment and the results recorded clear significant differences.

Table 2. Screening of Pseudomonas isolates for blue-green pigment production in waste-oil-supplemented nutrient broth after 48 h of incubation at 30 °C.

Isolate code	Source of isolation	Blue-green pigment production (µg/ml ±SD)
P. Dg	Agricultural drainage water sample from Dakahlia governorate	3.47 ±0.075b
P1	Soil polluted by gasoline from Dakahlia governorate	2.13 ±0.115c
W1	Nile river water sample from Dakahlia governorate	0.94 ±0.090e
W2	Nile river water sample from Dakahlia governorate	1.11 ±0.090e
W3	Nile river water sample from Dakahlia governorate	1.72 ±0.205d
W4	Irrigation water sample from Dakahlia governorate	5.74±0.271a
M1	Minced meat sample	1.60 ±0.135d
M2	Minced meat sample	1.03 ±0.115e

Data show that the bacterial isolate (W4), which is isolated from irrigation water, show a clear superiority in pigment production over the other isolates, as it produced 5.74 µg/ml, while isolate (P. Dg), which isolated from agricultural drainage water came in second place, producing 3.47 µg/ml of blue-green pigment. On the other hand, the isolate (W1) which isolated from Nile River was the least pigment producer, recording 0.94 µg/ml which is a very low amounts of blue-green pigment.

Identification tests were performed in vitro on the isolated bacteria to confirm that they belong to the genus *Pseudomonas*. Tests includes morphological tests (bacterial shape and motility test), and biochemical tests as gram staining, oxidase production, catalase test, gelatin liquefication, and lactose fermentation. Also isolates were tested for their ability to grow at 42°C as shown in table (3). The results of some tests were consistent with the characteristics of the genus *Pseudomonas*, as all isolates were short, Gram-negative, motile rods. All isolates also produced catalase and had the ability of gelatin liquefication. Data also show that all isolates do not have the ability of lactose fermentation, as it is a characteristic associated with the genus *Pseudomonas* and through which it can be distinguished from similar genera such as the coliform group species. Data also appeared that all isolates could produce oxidase except the isolate W4. The cultivation of isolates on 42°C could help in the differentiation of *Pseudomonas* sp., as *Pseudomonas aeruginosa* has the ability to grow at 42°C, while other *Pseudomonas* species do not grow at the same temperature.

From the results in Table 3, it can be confirmed that the selected isolates belong to the genus *Pseudomonas*. It can also be confirmed that the isolates; P. Dg, W2, W3, M1 and M2 could be *Pseudomonas aeruginosa*, as it is among the other isolates that can grow at 42°C.

Table 3. Some morphological and biochemical Characteristics of selected blue-green pigment producing Pseudomonas isolates:

Isolate code	Gram stain	Motility	Oxidase production	Catalase Test	Gelatin liquefaction	Lactose fermentation	Growth at 42°C
P. Dg	G-ve rods	Motile	+	+	+	-	+
P1	G-ve rods	Motile	+	+	+	-	-
W1	G-ve rods	Motile	+	+	+	-	-
W2	G-ve rods	Motile	+	+	+	-	+
W3	G-ve rods	Motile	+	+	+	-	+
W4	G-ve rods	Motile	-	+	+	-	-
M1	G-ve rods	Motile	+	+	+	-	+
M2	G-ve rods	Motile	+	+	+	-	+

The data presented in Table 4 illustrate the effect of different media, with various additives, on blue-green pigment production by the selected *Pseudomonas* isolates. The results presented in Table (4) indicate a significant variation in microbial pigment production among the different culture media. The findings revealed that King's medium was the most effective for pigment production compared to nutrient broth and Bushnell Haas media with all tested *Pseudomonas* isolates. On the other hand, the results demonstrated that the supplementation of nutrient broth and Bushnell Haas media enhanced pigment production. The addition of waste oil and glycerol to the nutrient broth medium resulted in significant increases in pigment production compared to nutrient broth medium without additives by all *Pseudomonas* isolates. Similarly, the enrichment of Bushnell Haas medium with peptone led to a marked enhancement in blue-green pigment production in all *Pseudomonas* isolates, compared to the unsupplemented Bushnell Haas medium.

Table 4. Effect of different media on blue-green pigment production by selected Pseudomonas isolates:

Isolate code	Nutrient broth	Nutrient broth + Waste-oil	Nutrient broth+ glycerol	Kings A Medium	Bushnell-Haas medium	Bushnell-Haas medium + peptone
P. Dg	0.311±0.006d	3.21±0.12a	3.34±0.03a	2.12±0.14c	0.11±0.02e	2.36±0.12b
P1	0.092±0.008d	2.34±0.07a	2.36±0.09a	1.86±0.06b	0.17±0.03d	1.54±0.13c
W1	0.033±0.009d	1.01±0.04a	0.98±0.11a	0.54±0.03c	0.02±0.01d	0.87±0.06b
W2	0.042±0.005e	1.05±0.08a	1.21±0.11a	0.84±0.09c	0.05±0.02e	0.68±0.1d
W3	0.061±0.004e	1.84±0.07b	2.14±0.07a	1.47±0.1c	0.06±0.01e	0.64±0.08d
W4	0.97±0.05e	4.98±0.26a	3.68±0.17b	2.65±0.017c	0.18±0.06f	1.54±0.07d
M1	0.077±0.012e	1.57±0.06b	2.02±0.15a	1.32±0.09c	0.10±0.03e	0.47±0.06d
M2	0.081±0.014d	1.21±0.11b	1.74±0.19a	1.57±0.12a	0.03±0.01d	0.83±0.06c

The *Pseudomonas* genus is considered a widespread bacterium that can be obtained from various sources and which widely produces a variety of extra-cellular pigments (El-Fouly *et al*, 2015). In this work, the blue green pigment producers of *Pseudomonas* were isolated from different ecological sources to study the production of the pigment. We focused on the effect of media and supplements on the production of blue-green pigment. We have found that the highest pigment production with different media were achieved by using waste-oil or glycerol as supplements to the nutrient broth medium, compared to the other tested conditions, where the isolate W4 recorded the highest pigment production among all treatments in the experiment when waste oil was used as a supplement to the nutrient broth medium, reaching a value of 4.98 µg/mL**.** In agreement with our results, Elbargisy, (2021) reported that using king's medium outperformed nutrient broth medium in the production of blue-green pigment with *Pseudomonas auroginosa*.

Gahlout *et al*., (2021) used nutrient agar medium for the production of pyocyanin from *Pseudomonas auroginosa* as it consists the most suitable nutrients for the pigment production. Also, they found that addition of glycerol to nutrient agar medium enhanced the pyocyanin dye by *Pseudomonas aeruginosa* compared with control medium.

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التحري عن إنتاج الصبغة الزرقاء-المخضرة بواسطة عزلات ***Pseudomonas***

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الملخص

تم عزل ثمانية عزلات تنتمي إلى جنس سيدوموناس من مصادر بيئية مختلفة، بما في ذلك التربة والماء والمواد الغذائية، واختبارها لإنتاج الصبغة الزرقاء المخضرة. أظهرت جميع العزلات المختارة إنتاجًا جيدًا للصبغة الزرقاء المخضرة ، مع ثلاث عزلات أظهرت إنتاجية عالية، حيث بلغت ٢,١٣ و٣,٤٧ و٥,٧٤ ميكروجرام/مل عند زراعتها في وسط غذائي مدعم بـ ١٪ من مخلفات الزيت. في محاولة لاختيار وسط مناسب لإنتاج الصبغة الزرقاء المخضرة، تم اختبار ستة أوساط مختلفة مع جميع العزلات. تشير البيانات التي تم الحصول عليها إلى وجود تباين كبير في إنتاج الصبغة الميكروبية بين الأوساط البيئية المختلفة. كشفت النتائج المتحصل عليها أن بيئة كينج كانت الأكثر فعالية لإنتاج الصبغة مقارنة ببيئة البويون المغذي وبيئة بوشنل هاس مع جميع عزلات السيدوموناس المختبرة. من ناحية أخرى، أظهرت النتائج أن تدعيم بيئة البويون المغذي وبيئة بوشنل هاس عزز إنتاج الصبغة. أظهر ت بيئة البيويون المغذي إنتاجية عالية مع جميع العزلات عند تدعيمها بمخلفات الزيت أو الجليسرول. تم تحقيق أقصى إنتاج للصبغة من قبل العزلةW4 ، حيث بلغ ٤,٩٨ ميكروغرام/مل عند زراعتها في بيئة البويون المغذي المدعمة بمخلفات الزيت.