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Production Pyocyanin from *Pseudomonas Aeruginosa* and the Inhibitory Effect of the against a Number of Pathogenic Bacteria

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Abstract

The pyocyanin stain was extracted from *Pseudomonas aeruginosa* K3 isolated from several food sources, and diagnosed using the HPLC device, and the biological efficacy of the produced pigment was tested on a number of pathological microorganisms.

The results of the study showed that there is sensitivity of pathological bacteria against the *pseudomonas aeruginosa* stain, andits refer to indicated an inhibitory effect for T2 treatment for all types of test bacteria.

The T2 treatment (the sample to which the raw pyocyanin e extract was added by 2%) had the highest inactivation by 28, 25 and 20 mm for *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus*, respectivelyand the least inhibitory was the direction of *Enterococcus faecalis*and*Salmonella typhimurium* reaching 9 and 5 mm respectively. While the comparison treatment T1 (using chloramphenicol solution) showed an inhibitory effect against the test bacteria, and it was 30 mm against *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus*respectively and by 26 mm was the direction of *Enterococcus faecalis*and*Salmonella typhimurium*, respectively.

Keyword: ***pseudomonas aeruginosa*** **,pyocyanin , HPLC, pathogenic bacteria**

Introduction

P. aeruginosa has received the increasing attention of researchers due to its great importance in the fields of medicine, food technology, environmental microbiology, plant pathology, etc. (Dabboussi, *et al.*, 2002) *P. aeruginosa* produces a variety of dyes as secondary metabolites, which play an important role. In interactions between *Pseudomonas* species and other organisms. Four different main pigments produced from *P. aeruginosa* have been described, namely, pyocyanin, fluorescein, pyroprine, andbiomelaninThe pyocyanin dye is a bluish green pigment soluble in chloroform produced by the active media of *Pseudomonasaeruginosa*, these dyes are produced independent of season and geographical conditions (Marrezand Mohamad, 2020),Zhao *et al.*, 2014).).

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Liu *et al.*, (2011) indicated that pyocyanin synthesis is affected by the sources of carbon and nitrogen in the growth medium, but most nutrients support pyocyanin production as long as the phosphate ion concentration is low and there is sufficient sulfate ion. Synthesis of this pigment also appears to be under controlIron concentration since adding iron to a medium containing low phosphates stimulates the synthesis of pyocyanin and related phenazine dyes by other types of bacteria. Physiological studies have shown that *P. aeruginosa* is resistant to toxicity of this compound with increased superoxide dismutase and lactase activity under conditions of pyocyanin production, (Price *et al.*, 2007 , Mezal, E. H*et al* 2020).

Baron and Rowe (1981) reported that the antimicrobial effect of pyocyanin is dependent on its concentration.

This study aimed to screening to pyocyanin productionand sing itsagainst a number of pathogenic bacteria.

Methods

1- Collections and Isolation, Purification andPreservation of Bacterial isolates

Samples were isolated from different isolation sources, as 10 ml of juices were suspended in 90 ml of distilled water in dilution bottles. As for samples of dairy products, jams, spoiled fruits and pickles, a sterile cotton swab was used in Swabs were taken from their surfaces and then placed in sterile tubes with 1 ml sterile distilled water, after which a decimal dilution series was performed.

2- Preparing the culture media for bacteria

1-2: Cetrimide agar

Preparation of the medium by dissolving 46.7 grams of Cetrimide acid in 1000 ml of distilled water containing 10 ml of glycerol, then sterilized with an oxidizer at a temperature of 121 ° C at a pressure of 15 pounds / inch for 15 minutes, then cooled to 45 ° C, and then add 15 mg. of Nalidixic acid, then mixed well and then poured into sterile dishes and left to harden and used in isolation and diagnosis of *P. aerugenosa*.

2-2: Solid Nutrient Medium (N.A)

Prepared according to the manufacturer's instructions by dissolving 28 grams of the medium in a liter of distilled water and sterilizing the autoclave. Use this medium in preserving the isolates in the form of slantes.

3-2: Nutrient broth (N.B)

Prepared by dissolving 13.0 grams in one liter of distilled water and according to the manufacturer's instructions and sterilizing with an autoclave. Use this medium in preserving the isolates and in preparing the bacterial inoculum.

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4-2: MacConkey Agar

Use medium prepared according to the supplier's instructions. Sterilize with an autoclave. Use this medium for phenotypic.

5-2: King A-Agar Bioscyanine Solid Dye Production Medium

Dissolve 46 gm of the medium in one liter, then add 10 ml of glycerol to the medium. Sterilize the medium, then let it cool down and pour into sterile dishes and keep at 4 ° C until use. This medium is used to detect the ability of *Pseudomonas aeruginosa* to form the blue-green pigment Pyocyanin (Murray *et al.* 2007).

6-2: King A-broth: The medium of production of liquid pyocyanin

Prepare the medium from gelatin, peptone (pancreatic) 20.0 g, magnesium chloride 1.4 g, potassium sulfate 10.0 g, and the final pH is 7.2 +/- 0.2 (at 25 ° C) and keep the prepared medium at a temperature below 8 ° C in the shade and store The dried powder is slightly yellowish-brown in color, in a dry place, and in airtight packaging at a temperature of 2-25 ° C. Then 41.4 g was dissolved in 990 ml of distilled water and 10 ml of glycerin added, sterilize the medium at 121 ° C for 15 minutes This liquid nutrient medium was used to detect the ability of *P. aerugenosa* to form a stain. Blue-green Pyocyanin (1985, MacFaddin).

7-2:: Muller Hinton Steel Center

The medium was prepared according to the instructions of the supplier company (Himedia) and used to test the inhibitory activity of pyocyanin stains.

3- Bacteria isolation and purification:

Samples were grown using a plate casting method as indicated by HarriganandMacCanse (1976), using solid medium between cetimide agar (CA) and the plates were incubated at a temperature of 37 ° C for 24 hours, isolates were purified by transferring the single developing colonies to dishes with CA streaked media by streaking with an inoculation needle. Loop and bis to ensure that pure colonies are obtained and incubated with the same previous conditions

4: Quantitative screening to determine the isolation of the most productive bacteria

1-4: production of pyocyanin pigment

Take 2 ml of the bacterial suspension of the growing cultures to make the final number $1x10^5$ cell / ml prepared in previously and transfer Into a 250 mL beaker containing 50 mL of sterile King A-broth liquid medium, and incubated in the vibratory incubator at 130 rpm at a temperature 37 c for 3 days, and the bacterial cells were removed .

Free of colored supernatants by centrifugation at 10,000 rpm for 10 minutes and sprayed through a 0.45 μm filter (Barakat*et al.*, 2015).

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2-4: Preparation of McFarland's Standard Curve Follow the method indicated by Collee *et al.*,(1996) .
used the straight-line equation from the standard curve For McFarland as in Figure (1-1).

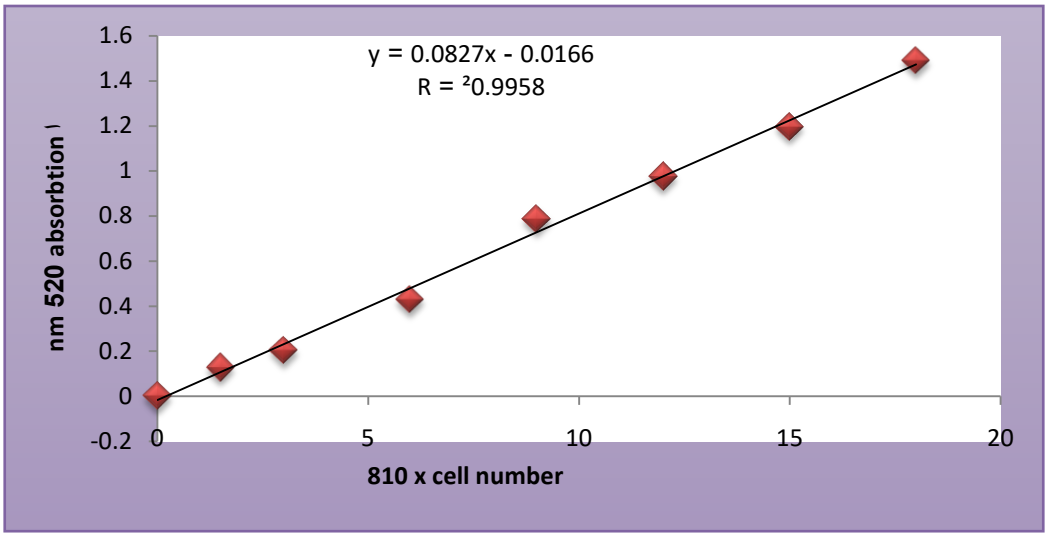

Figure (1-1) Standard Curve of McFarland to count the number of bacteria cells \ ml

5: Estimate the amount of pyocyanin produced from isolates

The method of dissolving the dye with chloroform solution was followed by DeBritto*et al.*, (2020) in the extraction of pyocyanin from the bacterial culture prepared in previously as it was centrifuged at a speed of 12,000 rpm for 20 minutes, then after that the supernatant was collected and mixed well with 4.5 Ml of chloroform and mixed with Magnetic Stirrer Mixer for 20 seconds to change the color, the biomass deposited at the bottom was dried at 75 ° C and the dried biomass was considered as a weight for each volume of 10 mL, (Chloroform dipped into the bottom of tube, make ensure Ensure that the supernatant moves with chloroform at the magnetic mixture, i.e., the supernatant moves with chloroform.
Chloroform changes color from green to blue). Samples were centrifuged at 10,000 rpm for 10 minutes at 4 ° C, after which 3 ml of the blue-layered solution stabilized at the bottom of the tube was transferred to another fresh set of tubes and 1.5 ml of 0.2 M hydrochloric acid was introduced each. In tube, and centrifuged for 10,000 min / min for 5 min, the resulting suspension containing the dye was stored at 4 ° C until further use. The obtained supernatant was used to measure the density of the crude pyocyanine extracted with different wavelengths in a spectrophotometer (Hitachi U2900). The intensity of the pyocyanin concentration (µg ml^{-1})) was calculated by multiplying the optical density (OD) value obtained at 520 nm.

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5- Bacteria diagnosis

The selective isolate that gave the highest efficacy of dye production was diagnosed through culture and microscopy tests, and some biochemical tests, as well as the Vitek2 diagnosis.

5-1: culture examinations

The culture characteristics of the growing bacterial colonies were studied on MacConkey agar and Cetrimide agar to diagnose the culture characteristics of the shape of the colonies, their size, color, aroma and their ability to produce pigments. Green is shown on citrimide culture medium which only allows growth of *P. aeruginosa*and prevents growth of other bacterial species (Levinson, 2016).

5-2: Microscopic examinations

a) The Gram stain test: The bacterial isolates were microscopically examined by taking a smears and stained with a Gram stain, and then examined under a microscope to observe its interaction with the dye as well as the shape and arrangement of the cells (Forbes *et al.*, 2007).

b) Mobility test: The mobility test of bacteria was performed using the hanging drop method referred to by McManus *et al.*, (1980) was placed in the middle of the vitreous cover, and then the slide was flipped over the cap and examined under a microscope with an oily lens to observe the movement of bacterial cells.

5-3: Biochemical tests

Biochemical tests were performed to diagnose P. *aeruginosa*:

a) The oxidase test

A single bacterial colony at the age of 18-24 hours is taken by a sterile wooden stick to a piece of filter paper, then we put two drops of the oxidase reagent on top of it, if the color change to a dark purple or violet color within 10 to 60 seconds is an indication of the production of oxidase (Procop*et al.*, 2016).

b) The catalase production test: The ability to isolate the production of the enzyme catalase was tested by taking a swab from a single bacterial colony at an age of 18-24 hours and placing it on a glass slide and then adding a drop of hydrogen peroxide solution H_2O_2 at a concentration of 3%, as the appearance of bubbles is evidence of production Catalysis (Taylor andAchanzar, 1972).

c) Growth at two temperatures (4 ° C and 42 ° C)

The bacterial isolates were planted by streaking and on the feeding medium and incubated at 4 ° C and 42 ° C for 7 days, and other tubes were inoculated to the same medium and used as a control treatment by placing them at a temperature of 22 ° C. The appearance of turbidity in the medium was an indication of the ability of the isolates. StolpandGadkari, 1981, on growth at the desired temperature.

d) Diagnosis using the Vitek 2 compact system .

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6: Pyocyanin production

6-1 :mthod to production

Take 2 ml of the bacterial suspension of the growing culture to make the final number 1 x10 ^{5} cell / ml previously prepared and transfer it to a 250 ml container beaker. On 50 ml of sterile Nutrient broth medium, it was incubated in a shaker incubator at 130 rpm at 37 C for 3 days. Remove bacterial cells free of colored supernatants by centrifugation at 10,000 rpm for 10 minutes and sprayed through a 0.45 mm filter (Barakat *et al.,* 2015, Dilfy, S. *et al* 2020).

6-2: extraction of pyocyanin

Initial experiments were conducted in the laboratories of the Agricultural Research Department / Laboratory of Biotechnology of the Ministry of Science and Technology to test the best method for extracting pyocyanin e, which gives the highest production of pyocyanin e and the biomass of the extract from the bacterial culture, and the extraction was done by following the method of dissolving the dye with chloroform solution mentioned by DeBritto, *et al.*, (2020) in the extraction of pyocyanin from the bacterial culture, as it was centrifuged at 12,000 rpm for 20 minutes, then the supernatant was collected and mixed well with 4.5 mL of chloroform. The Magnetic Stirrer was mixed with the Magnetic Stirrer for 20 seconds to change the color, and the biomass deposited at the bottom was dried at 75 ° C.

The dried biomass was considered as a weight for each volume of 10 mL, (chloroform sinks to the bottom of the tube, make sure that the supernatant moves with chloroform when the magnetic mixture, the chloroform changes color from green to blue). Samples were centrifuged at 10,000 r / min for 10 min at 4 ° C, then 3 mL of the solution with stabilized blue layers at the bottom of the tube were transferred to another fresh set of tubes and 1.5 mL of 0.2 M hydrochloric acid were introduced each. In tube, were centrifuged for 10,000 cycles and for 5 min, the resulting suspension containing the dye was stored at 4 ° C until further use.

The obtained supernatant was used to measure the density of the crude extracted pyocyanin with different wavelengthsin a spectrophotometer (Hitachi U2900). The intensity of the pyocyanin concentration (µg ml^{-1})) was calculated by multiplying the optical density (OD) value obtained at 520 nm.

6-3: Diagnosis of the pyocyanin stain of *Pseudomonas aeruginosa* using an HPLC

The pyocyanin pigment was determined using a high-performance liquid chromatography device (HPLC) of German origin and manufactured by (Sykamn) in the Environment and Water Laboratories / Ministry of Science and Technology to estimate the amount of pyocyanin e, whereas the carrier phase consisting of (acetonitrile: distilled water) was used (80:20). And a (C18-ODS) separation column (25cm * 4.6 mm) used a UV-Vis detector in a wavelength (450 nm), as the pyocyanin dye was extracted from the bacterial culture in paragraph (6), purify the extract through a 0.25 µm membrane filter and centrifuge the filtrate,

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Standard pyocyanin was prepared at different concentrations (12.5, 25, 75, 100 and 125) µg, flow rate of 1 ml / min, syringe volume of 20 micrograms (Madhurimaand Pooja 2013).

7: Detection of the inhibitory activity of the extract of pyocyanin from*Pseudomonas aeruginosa* against the test
BacteriaInhibition of pyocyanin was tested in *Pseudomonas aeruginosa* against pathogenic microorganisms, *Escherichia coli*, *Bacillus cereus*, *Salmonella typhimurium*, *Enterococcusfaecalis*and S*taphylococcus aureus* obtained all from Engineering Sciences / University of Baghdad. According to the method reported by Awad (2016), the drilling method was followed with a cork drill with a diameter of 0.6 mm to make three holes in each plate. The pyocyanin dye was prepared at a concentration of 2% and was dissolved in 100 ml of DMSO (Di Methyl Sulfoxid) and a concentration of 10 µl was placed in each pit. As for the third hole, a chloramphenicol solution at a concentration of 0.1 was added as a comparison sample.
Then, 1 ml of the test bacteria was seeded on the surface of the medium and spread it with a sterile glass rod, and the dishes were incubated at 30 C for 24 hours. Then the diameter of the inhibition zone was measured using a ruler and the method was performed with three replications for each type of bacteriaandusing the food medium Mullerhenton.

8: Statistical analysis
The statistical program Genstst12 was used in data analysis to study the effect of different factors on the studied traits according to a complete random design (CRD), and the significant differences between the averages were compared with the Least Significant Difference-LSD test.

3: Results and discussion
3-1: Isolation of *Pseudomonas aeruginosa*
The study included collecting 45 samples from several different sources that included dairy products, jams, juices, spoiled fruits and pickles taken from different locations in Kufa, Al-Mishkhab, Al-Salam neighborhood, the old city, Al-Razawiya, Al-Manaraand Al-Qadisiyah for the period from 01/10/2019 to 15 / 5/2020. After making the final diagnosis of the samples, 12 greenish blue isolates were obtained in this study from 18 samples as shown in Table (1-3).
The table shows that 5 colored isolates obtained from curd milk (taken from dairy products for Kufa) and 5 isolates from Arab cheese (taken from the popular market in Al-Salam neighborhood), while two isolates were from pickles (taken From the popular market in Qadisiyah) , no colored isolates were obtained from them, and they were neglected. Thus, milk, Arab cheese and pickles taken from different sources are the main source on which the isolation process relied on in this study.
This result is consistent with what was reported by Al Habib and Al Jabbouri (2017) that the types of bacteria and fungi isolated from local soft cheese in Al Qadisiyah governorate, and the results of the diagnosis of Vitek2 showed four types of bacteria which are E. coli, *Pseudomonas aeruginosa* ,*Staphylococcuslentus*and*Kocuriakristinae*. De Oliveria*et al.,* (2016) emphasized that

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raw milk provides a physical environment which is conducive to the reproduction of a wide range of microorganisms, including the cold-loving bacterial group Pseudomonas (mostly individuals of the genus *Pseudomonas*) that contaminate the milk. During its collection or processing.

Table (1-3): Sources of isolates of *Pseudomonas aeruginosa* , the number of colored isolates obtained, their symbol, place of isolation, and the percentage according to the source of isolation.

Percentage	Isolationareas	Isolation symbol	The number of colored isolates	The number of samples	Isolation sources	
83.3	**Al -Kufa**	**K1-K2-K3-K4-K5**	**5**	**6**	Sour milk (dairy products**)**	1
83.3	**AL-Salam governorate**	**C1-C2-C2-C4-C5**	**5**	**6**	Arab cheese dairy products	2
33.3	**Qadisiyah governorate**	**J1-J2**	**2**	**6**	Pickles	3
66.7			**12**	**18**	**Grand total**	

3-2: Characteristics of Pigment-producing isolates of *Pseudomonas aeruginosa*

The twelve isolates were subjected to a set of microscopic and culture tests and some biochemical tests, which showed that they apply to the most important characteristics of *Pseudomonas aeruginosa* , which he mentioned; Sudhakar, *et al*, 2015; Al-Dahmoshi, 2013;Khalifa, 2019) and as shown in Table (1-3).

3-2: culture characteristics

The culture characteristics of isolates were studied from the previous steps, as they were grown on the solid nutrient media (Nutrient agar) as well as the medium (CA) cetimide agar and incubated at a temperature of 37 ° C, as the bacterial colonies were formed in the greenish yellow color with irregular edges and a mucous consistencyand it has a distinctive smell, as most of these colonies produce the pigment Pyocyanin, which is a greenish-blue color. Figure (3-1)

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These traits are identical to the characteristics of *Pseudomonas aeruginosa* mentioned by the researcher. Sudhakar, *et al.*,2015).

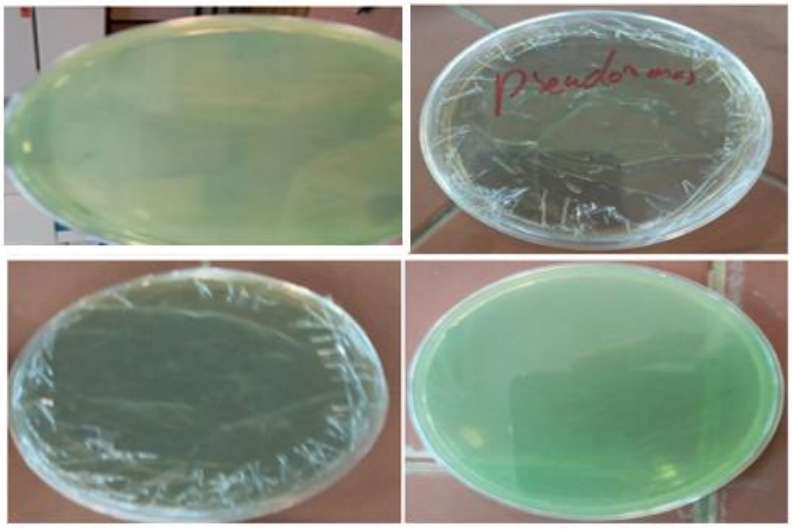

Figure (1-3) culture of *Pseudomonas aeruginosa* on Nutrient agar

3-3:Microstructure

The microscopic characteristics as shown in table (2-3) for the cells after staining them with a cream dye and examining them with the oil lens, that they are small single and sometimes double and negative Cram-stained RMCs, not forming non-spore-forming spore cells (D-Aloshi 2013). The results are in agreement with (D-Aloshi 2013).

3-4: Biochemical properties

Based on the aforementioned agricultural and phenotypic characteristics, it can be concluded that C4, K2 and K3 isolates may be selected and have undergone some biochemical tests. These tests included, testing for oxidase and catalase production, growth at temperatures (4 and 42) and a diagnosis of Vitek2.

All isolates have shown positive results for each of the examination, which Oxidase between the possession of bacterial enzyme cytochrom oxidase, and examine Catalase which explained the bacteria's ability to break down hydrogen peroxide to water and gas, bacteria and showed their ability to grow at a temperature of 42 ° Cand its inability to grow at a temperature of 4 ° C, so the result of the examination was negative, and these results are consistent with what TadessandAlem (2006) andTodar (2011) show. Results of these tests As for the results of the physiological and biochemical examinations conducted using the Vitek2 device, it confirmed that the C4 isolate selected was *Pseudomonas aeruginosa* .

Shows the test results included in the Pseudomonas Genus Diagnostic Card. Al-Habib and Al-Jabbouri (2017) explained that the diagnosis of *Pseudomonas aeruginosa* , isolated from local

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soft cheese, was confirmed in the local markets in Qadisiyah Governorate, using the Shimadz-Japan device, and using the diagnostic kit for the device to diagnose bacteria.

Table (2-3) Some other biochemical and diagnostic tests.

Result	The test
+	**Oxidase production**
+	**Catalase production**
-	**Growth at 4 ° C**
+	**Growth at a temperature of 42 ° C**

3-5: Quantitative screening to determine the isolation of the most productive bacteria

The screening process was carried out using the sterile Nutrient broth by the method of fermentation of the liquid state, and to determine the most efficient isolates from the above-selected isolates in producing pyocyanin , the efficient isolation was chosen on the basis of the dry cell biomass weight and the amount of pigment produced. From the results in Table (3-3) the variation in the ability of isolates to produce pyocyanin and that the highest concentration of the pigment obtained was 5.25 g / ml^{-1} for isolate K3 after a period of 72 hours, when the weight of the dry biomass of the isolate reached 52000 mg / liter. ,
The results of the statistical analysis showed that there are significant differences (0.05) between the isolate K3 and the other isolates, and based on the results obtained from the quantitative screening, the isolate K3 was adopted to complete the current study experiments after subjecting it to culture, microscopic and biochemical tests using the Vitek2 device. Figure (4-8) shows the isolation of K3 at the end of the 3-day fermentation process.

Table (3-3) shows the results of the quantitative screening of the produced bacteria isolates

pyocyanin concentration intensity (µgml^{-1})	dry biomass weight mg / l	dry biomass weight g / l	Fermentation time	العزلة
3.50	10000	10	hours 24	**C4**
4.75	41000	41	hours 48	**K2**
5.25	52000	52	hours 72	**K3**

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*0.25		*15	LSD0.05

isolation

This result confirms that the liquid medium King broth A works to develop the pigment pyocyanin (the blue pigment) and prevents the formation of the fluorescein pigment, as gelatin and peptone provide the basic nutrients nitrogenous, carbon and sulfur and contain a low percentage of phosphorous to reduce the inhibitory effect on the production of pyocyanin , Glycerin acts as a C-source, and potassium sulfate and magnesium chloride are necessary to activate pyocyanin e production, (MacFaddin,.1985).

Also, Barakat *et al.,* (2015) reported that Marine *Pseudomonas aeruginosa* OSh1 isolate produced the highest yield of bluish green pigment as the dry biomass reached 26 g / ml after fermentation on liquid King A broth. (Jayaseelan*et al.,* 2010) also showed that the bacterium *Pseudomonas aeruginosa* builds colonies surrounded by a bluish green area due to the production of the pyocyanin pigment, although the medium (King A broth) may produce red pigments (biorbin) orYellow green (bioveridine, fluorescein) or brown (biomelanin) and mask the pyocyanin pigment. The colored dyes are extracted by adding 0.5-1 mL of chloroform to the liquid medium and shaken for a few minutes until the pyocyanin diffuses, making the solvent blue. After that, some drops of hydrochloric acid are added and a rapid change in color from blue to red appears, and this confirms the presence of pyocyanin .

3-6 :Diagnosis of pyocyanin stain of *Pseudomonas aeruginosa* with High Performance Liquid Chromatography (HPLC)

Figure (2-3) shows a profile of using HPLC to diagnose pyocyanin produced from the select isolate Pseudomonas sp. K3 and its comparison with the standard pyocyanin profile and according to the approved separation conditions. It was found that the holding time of the standard pyocyanin e is 14.3 minutes, which corresponds to the retention time of the first peak of the *Pseudomonas* sp. Isolation profile. K3, which appeared at the same time, 14.3 minutes, confirming that the pigment produced from these bacteria is pyocyanin ,and the other peak appears at 24.4 and 25.2 minutes representing other types of pigments produced by these bacteria.

We conclude from this result that the use of HPLC technology was highly efficient in diagnosing pyocyanin produced from the selective isolate Pseudomonas sp. K3, and this result confirms what was stated by Hernández-Almanza, et al., (2017). There are many mechanical, chemical and enzymatic methods used separately or in combination to extract microbial pigments. But they all follow the same basis and are known to researchers and have proven to be suitable for extracting pigments.

Barakat *et al.,* (2015) also indicated that the extracted compound represented by *P. aeruginosa* OSh1 strain was analyzed with a GC-MS device to confirm the presence of the pyocyanin stain, and the results of this technique revealed a peak of molecular ions of 196 µg / l, which appeared

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in The holding time is approximately 20,758 minutes. This peak was the apex of a standard pyocyanin dye

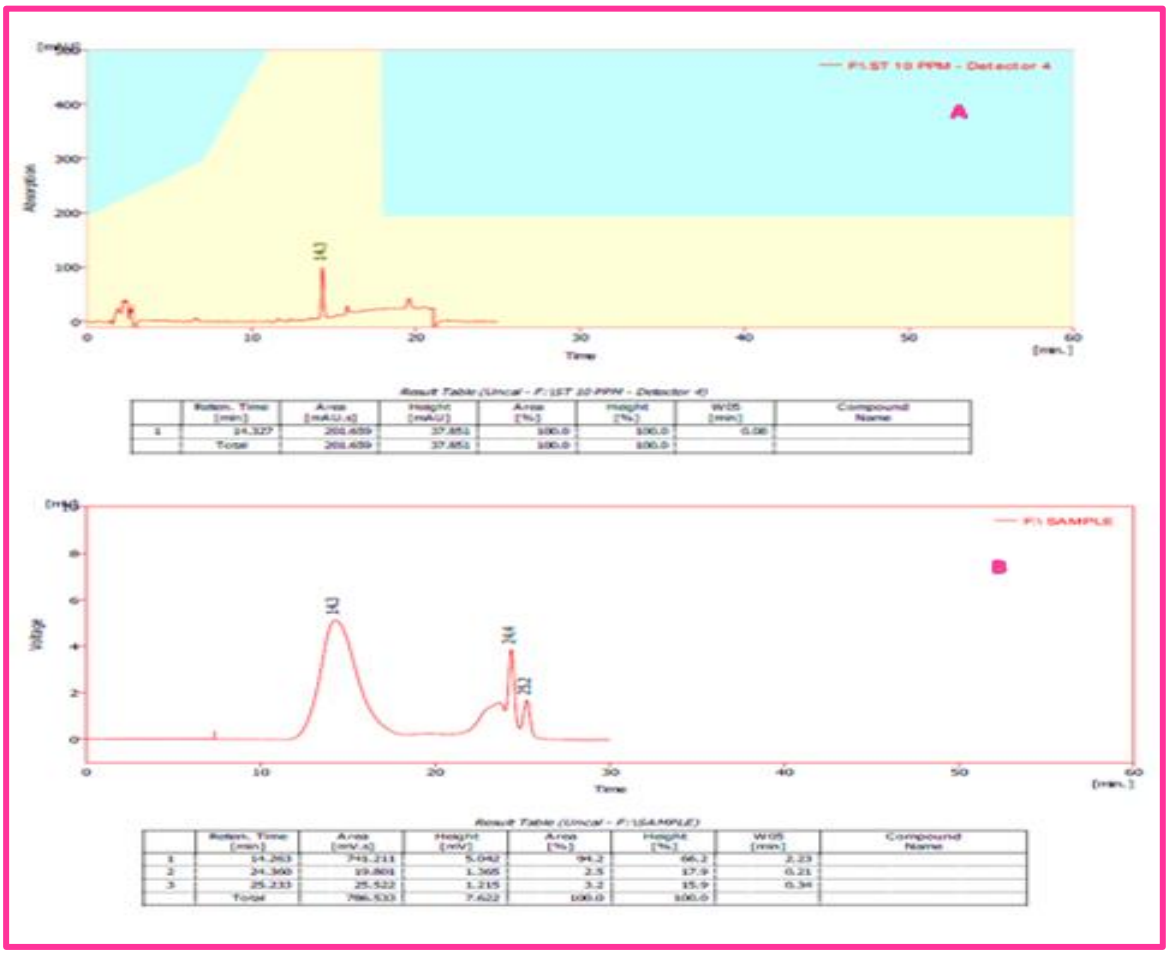

Figure (2-3) shows the absorbance profile using an HPLC device at 450 nm wavelength: (A) standard pyocyanin (B) pyocyanin produced from *Pseudomonas* sp. K3

4: Detection of the inhibitory activity of the extract of pyocyanin from*Pseudomonas aeruginosa* against the test

The results of Table (4-3) showed an inhibitory effect of T2 treatment for all types of test bacteria. Treatment T2 (the sample with added 2% raw pyocyanin extract) showed the highest inhibition was 28, 25 and 20 mm for *Escherichia coli*, *Staphylococcus aureus*and*Bacilluscereus*, respectively, and the lowest inhibition was the direction of *Enterococcus faecalis*and Salmonella typhimrum, reaching 9 and 5 mm, respectively. While the comparison treatment T1 (using chloramphenicol solution) showed an inhibitory effect on the test bacteria, and it was 30 mm towards *E. coli*, *S. aureus*and*B. cereus*bacteria.

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On the other hand, 26 mm were the orientation of *E. faecalis*and*S. typhimrum*, respectively. From the results of this study it is noticed that the sensitivity of bacteria to the direction of the extract differs according to the type of bacteria, and the reason is due to the nature of the structure of the outer wall of the bacteria, as the peptidoglycan layer is thicker in the wall of bacteria positive for the Gram stain, while the bacteria negative for the Gram stain outside the peptidoclycan layer on a layer of The lipid polysaccharides of phospholipids and a lipoprotein, as these three compounds keep the cell from entering chemicals into the cell, and these together, Cram-negative bacteria acquire resistance that differs in range from Cram-positive bacteria (Fox, 2014). This corresponds to several studies that reported a difference in the antimicrobial activity of animal and plant extracts depending on the type of animal and the type of microorganism and that there is a significant relationship between the chemical composition of the bacteria and the inhibitory activity of the pyocyanin extract produced from bacteria at a significant level of 0.05).

These results were in agreement with what Marrezand Mohamad (2020) indicated that approximately 90-95% of the antimicrobial inhibitors of *P. aeruginosa* strains were due to the production of the water-soluble secondary metabolite pyocyanin . It showed antagonistic efficacy against pathogenic bacteria such as *Salmonella paratyphi, E. coli, Klebsiella pneumonia.*

Rahman et. al (2009) , indicates that the pyocyanin stain isolated from P. aeruginosa 4B showed antibiotic activity against various pathogens and food spoilage bacteria such as *Listeria monocytogens*and*Bacillus cereus*. Its antimicrobial activities. Fontouram, *et.al.* (2009) also revealed that pyocyanin from*P. aeruginosa* DSO-129 has an antimicrobial effect on organisms such as *S. aureus, Staphylococcus epidermis, Bacillus subtilis, Micrococcusluteus*, and*Saccharomyces cerevisiae*. Also, many studies have reported on the antibiotic activity of pyocyanin against various pathogens, as the pigment resulting from the strain has shown very effective activity against organisms such as *E. coli,Acinetobacter, S. aureus, and Streptococcus pneumonia*, Sweden, 2010).

Also Sudhakar, *et.al.* (2015) found that pyocyanin from *P. aeruginosa* SU1 against *E. coli, S. aureus and Proteus sp.* and *Klebsiella sp*. and the *Pseudomonas*sp maximum activity was seen against *E. coli, S. aureus, and Proteus* sp. and*Klebsiella sp.*

Table (4-3) Detection of the inhibitory activity of *Pseudomonas aeruginosa* produced pyocyanin extract against test bacteria.

Diameter of the inhibition zone (mm) for pyocyanin					**Transaction**
S.aureus	*E. faecalis*	*S. typhimurium*	*B.cereus*	*E. coli*	
30	**26**	**26**	**30**	**30**	**T_1 Chloramphenicol**

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25	**9**	**5**	**20**	**28**	**T_2 %2**
2.3	**11.1**	**13.4**	**8.6**	**1.8**	**T-test**

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