MOLECULAR DETECTION OF PSEUDOMONAS AERUGINOSA ISOLATED FROM MINCED MEAT AND STUDIES THE PYOCYANIN EFFECTIVENESS ON PATHOGENIC BACTERIA

D. A. Qasim
Market Researches and Consumer Protection Center/ University of Baghdad
doaalani@yahoo.com

ABSTRACT
This study was aimed to collected Minced meat from the local markets in Baghdad governorate during 2018, and examined for the presence of Pseudomonas aeruginosa, in order to extract and purify pyocyanin and examined it as an antimicrobial activity against pathogenic bacteria in foods. Fifteen isolates were isolated from 50 samples and identified as P. aeruginosa using the API20E system and finally confirmed with PCR using 16SrRNA gene. Four tested media were used for the production of pigment after incubation within 72 h, One strain which given a vigorous pigmentation was chosen and extracted with chloroform and HCI then analyzed with Gas chromatography (GC-Mass) which showed a sharp peak at the time of acquisition of 27.13 minutes at the chromatographic analysis recognized with mass spectrometry as Hemipyocanin (alpha-hydroxy phenazine) which produced molecular ion with intensive peak at 205 m/z. Agar well diffusion technique was applied for estimating the antimicrobial activity of purified (pyocyanin) with variable concentrations (25, 50, 75 and 100 mg/ml) which monitored toward Gram-negative and Gram-positive bacteria that isolated of minced meat. Escherichia coli and staphylococcus aureus was the most affected with pyocyanin were followed by Serratia marcescens and Klebsiella sp at the same level. While Enterobacter sp, Bacillus cereus, Proteus mirabilis, and Proteus vulgaris showed intermediate sensitivity, the Pseudomonas fluorescens was shown low sensitivity to pyocyanin.

Keywords: pyocyanin purification, gas chromatography, inhibition zones, 16SrRNA Gene.

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INTRODUCTION
The shelf life of foods is identified being the period when the food quality remains satisfying within severe conditions of storage, distribution, and display. Spoilage is the method in which food has degenerated and turns into unacceptable for humans being or its quality is diminished turning food improper for selling or consumption (13). Several bacterial isolates which are particular as spoilage organisms (SSO) of meat, fish and poultry that can be identified through the ability for analyzing the nitrogenous components and generating the volatile compounds such as (ketones, esters, and aldehydes) that responsible for the flavor that will be formed at the point of spoilage. Some organisms primarily cause a change in sugars by oxidation and producing alkali and other organisms produce a fluorescent pigment (3). From the total of microflora, Pseudomonas spp. may represent the minority at the beginning of shelf life of the food then become dominant at the end. Phenazines are comprised the most significant extracellular pigments that produce from genus Pseudomonas, P. aeruginosa which is rod shape, aerobic and a Gram-negative opportunistic pathogen. Pseudomonas aeruginosa has a distinctive feature through synthesized of the blue-green, chloroform-soluble compound called pyocyanin (1-hydroxy-s-methelphenazine) (9). A number of virulence factors are secreted by P. aeruginosa which is considered the physiological and pathological effects of these bacteria. Of these virulence factors, Pyocyanin is phenazine oxidation pigment with low-molecular-weight that produced by P. aeruginosa (14). The Pyocyanin production is regulated by sensing the quorum, which involves a cell-dependent synthesis of signaling molecules that modify the expression of virulence genes (19). In spite of the fact, that pseudomonad has repeatedly been described for its pathogenicity; the capability of these microorganisms to produce antimicrobial pigment has opened the opportunity to an application of this agent as a biological regulator (19). Pyocyanin has antimicrobial activity toward wide different microorganisms, which may assist P. aeruginosa through eliminating competing microorganisms; pyocyanin serve as an antimicrobial agent, selectively inhibitors for gram-positive and gram-negative bacteria rather than Pseudomonas spp. The redox-active phenazine compound (Pyocyanin) which kills bacterial cells by the production of reactive oxygen intermediates. P. aeruginosa resists pyocyanin because of the limited redox cycling of this compound and that under conditions favoring pyocyanin production; catalase and superoxide dismutase activities are increased. Researchers created numeral and substantial modern antimicrobial agents within the latest thirty years; simultaneously the resistance of bacteria to the antimicrobial agents has more progressed. The aim of this study is to isolate various isolates of P. aeruginosa from minced meat with purifying and discriminate the pyocyanin pigment by conventional methods and study the pyocyanin properties as antimicrobial activity toward some pathogenic bacteria.

MATERIALS AND METHODS
Sampling
This survey was carried out during 2018, 50 fresh minced meat samples were randomly collected from Baghdad supermarkets, Iraq. The samples were stored in the ice box while transport to the University of Baghdad/ laboratory of market research and consumer protection center for examination.

Isolation with identification of P. aeruginosa and target bacteria from clinical samples
P. aeruginosa is isolated out of minced meat specimens: blood agar, nutrient agar and Pseudomonas Cetrimide Agar (OxoidTM) and selective media for each microorganism. In beginning; Twenty-five gram of freshly minced meat specimens have been homogenized within peptone water (225 ml), samples were later cultivated on selective agar media through streaking and pour plate technique and incubated at 35 °C within 48 h. (16). Observed the distinguishing pigmentation and compare the physiological and microscopic aspects with biochemical characters of the isolates through the official description presented in “Bergey's Manual of Determinative Bacteriology”, that were recognized as P. aeruginos then the positive isolates has been confirmed with (API 20E).

1200
Brain heart infusion agar was used to preserve the pure strains as slants form (16).

**DNA extraction**

A genomic DNA of \textit{P. aeruginosa} was extracted for PCR amplification depending on company instruction Kit of DNA (G-spinTm, INtRON, Korea). Bacterial culture was transported to the microcentrifuge tube and Centrifugation at 13,000 rpm for one min, a buffer of Lysozyme was insert into the (centrifuge tube) the lysozyme was completely dissolved by using a vortex, the lytic when finished, the centrifuge was repeated twice and washed with buffer, the extracted DNA was saved at 4°C until use. 1.0 % agarose gel was used to Electrophoresis the purified DNA. Five microns of DNA was combined beside three μl loading dye of bromophenol blue then photos were taken through using U. V. light 350 nm (Sambrook and Russell, 2001).

**Detection of \textit{P. aeruginosa} using 16S Rrna**

| Target gene | Primer name | Sequence (5'–3') | Product size(bp) |
|-------------|-------------|------------------|------------------|
| 16S rRNA gene | \textit{Pseudomonas-F} | 5'-CTACGGGAGGCAGCAGTGG-3' | 150 |
| \textit{Pseudomonas-R} | 5'-TCGGTAACGTCAAAACAGCAAAGT-3' | | |

**Extraction, purification, and characterization of the pigment produced by \textit{Pseudomonas} isolates**

The isolates of \textit{p.aeruginosa} those given a vigorous pigmentation were selected and grown with the broth of \textit{Pseudomonas} at 37°C within 48 h for generation of pigment. The broth culture rich with Pigment was later centrifuged by (10,000 rpm within 15 min) then the supernatant was accumulated, later filtered within filter membrane pore sized (0.45μm) and applied as the crude extract (7). (Chloroform and HCl) was adopting for Extraction of pigment from the crude extract, Chloroform was combined within the broth culture at the proportion of (2:1). The extract was stirred well by utilizing a shaker for 2 min, then divided out into two discrete layers, one of them was the pigment (a blue solvent layer), and the other was a residual material of culture. The blue layer was accumulated, later solution of 0.1N HCl (20% for the blue layer’s volume) was combined then vortexed, then generated an upper pink acidified layer. The pink layer was later neutralized by Tris-Base then the neutralized layer was treated with chloroform again. The entire technique was repeated for numerous times to turn into purified pigment (7).

**GC-MS Chromatograph of \textit{Pseudomonas aeruginosa} pyocyanin**

Pyocyanin was analyzed by using gas chromatography (GC-Mass) spectrophotometer with autosampler system (PerkinElmer/USA) this device provided with a carbowax (30*0.25mm ID) and (0.25μm thickness of film) capillary column (intercut DB5Ms. Japan). One μl of extracted Pyocyanin was autosampler inside the capillary column. The carrier gas (Helium) was adopted. Temperatures of Injector and detector were arranged at 280°C. The temperature of the column was programmed firstly at 40°C to 1 min and later expands to a 5°C rate per min at a terminal temperature of 290°C. Pigments were separated with at (96.1 Kpa) constant pressure and the flow of column 1.71 ml/min. Peaks have been recognized by
comparing the mass spectra versus the mass spectral database (7).

**Screening of P. aeruginosa pyocyanin as antimicrobial activity**

Antimicrobial activity of pyocyanin toward each isolated bacteria was prepared by using well diffusion technique on Mueller-Hinton Agar following aerobic condition, 100μl of bacterial suspension was poured on the surface of MHA spread by L- shape glass rod and left for 10 minutes to settle down the bacteria and 120μl of different concentrations (25,50,75 and 100 ppm) of purified pyocyanin was added to the prepared wells in the same plate and incubated at 37˚C for 24h-48h, the diameter of the inhibition zone was measuring around the wells which represent the antimicrobial activity of pyocyanin (6).

**Statistical analysis**

The program of Statistical Analysis System-SAS (18) was employed to perform the different factors in investigation parameters. The LSD (least significant difference) test has been employed to significant compare within the means of this investigation.

**RESULTS AND DISCUSSION**

**Isolation and identification of P. aeruginosa**

The Bacterial isolates that chosen from minced meat sample were cultivated on blood agar and MacConky agar medium, isolates which revealed positive hemolysis activity were elected and re-cultured on nutrient agar and selective agar, these bacterial isolates were identified morphologically and microscopically and the result shown that there are several isolates belong to several genera as in table (2). Out of 50 selected minced meat samples, only 15 strains have been predicted to be *P. aeruginosa*. While the other bacteria were (8 isolates for *Psedomonas fluorescens*, 24 for *Escherichia coli*, 13 for *Klebsiella sp.*, 12 for *Staphylococcus aureus*, 10 for *Bacillus cereus*, 15 for *Proteus mirabilis*, 9 for *Proteus vulgaris*, 11 for *Serratia marcescens* and 12 isolates for *Enterobacter sp*) were chosen as target bacteria. *P. aeruginosa* is human's opportunistic pathogen, relating to the *Pseudomonadaceae* bacterial family which is popular within the environment; in the clean water, soil, and contaminated food. It has also been widely isolated from fish, meat products and canned food (4, 5).

| Bacterial species isolated from 50 fresh minced meat | NO. of positive isolates | Percentage (%) of positive isolates |
|-----------------------------------------------------|---------------------------|-----------------------------------|
| *Pseudomonas aeruginosa*                             | 15                        | 30                                |
| *Psedomonas fluorescens*                             | 8                         | 16                                |
| *Escherichia coli*                                   | 24                        | 48                                |
| *Klebsiella sp.*                                     | 13                        | 26                                |
| *Staphylococcus aureus*                              | 12                        | 24                                |
| *Bacillus cereus*                                    | 10                        | 20                                |
| *Proteus mirabilis*                                  | 15                        | 30                                |
| *Proteus vulgaris*                                   | 9                         | 18                                |
| *Serratia marcescens*                                | 11                        | 22                                |
| *Enterobacter sp.*                                   | 12                        | 24                                |
| Total isolates                                       | 129                       |                                    |

Morphological and biochemical features confirmed that *Pseudomonas aeruginosa* is a smooth, large, and irregular bacterium, surrounded by bluish-green coloration with grape-like odor. All the isolates were aerobic, catalase positive, nitrate reduction positive, showed oxidative metabolism on Hugh Leifson medium and the ability to stain with gram stain appear negative when examining microscopically with rods shape, motile. The results of the biochemical characterization, determined by means of the API 20 E for *P.aeruginosa* in figure (1), those results are consistent with results that observed via (20) who identified *Pseudomonas aeruginosa* that isolates from food.
In the microbiology laboratory, *P. aeruginosa* considered a very common isolate and its identification by conventional biochemical or commercial kits or by automated means may lead to a somewhat expensive process of identification. On the other hand, 24 hours or more may be needed to carry out for identification, so the identification of the *Pseudomonas aeruginosa* genus was confirmed by tests with the specific primer of 16S rRNA by PCR. The ranges of purity of extracted DNA out of 1.7-2.350 nm U.V was used for visualized the extracted DNA followed by electrophoresis with 1% agarose gel by 70 volts within 30 min. The genomic DNA of isolates have been detected with 2.0% of agarose gel electrophoresis which dyed via red safe stain and electrophoresed in 70 volts about 1:30 hr, the 15 lanes in figure (2) have been captured with ultraviolet 350 nm (UV) transilluminator with size of band of (150) bp plus (100) bp as DNA ladder and this result was reported previously by (12) study and confirmed by (11).

The differentiation of the 16S rRNA gene permits comparison at the genus level between organisms of bacteria, as well as to classifying isolates at multiple levels. The 16S rRNA gene sequence was noticed by (15) who have analyzed of 5.0 isolates of *Pseudomonas* contain 99% nucleotide sequence comparable to *P. aeruginosa* despite its varied considerably in pyocyanin generation.

### Production of pyocyanin

During growth of *P. aeruginosa* on the four tested media; blood agar, nutrient agar, Muller
Hinton agar, and Mac Conkey agar, it was concluded that there are various nutritional media can be utilized by *P. aeruginosa* for biosynthesis of pyocyanin. During this investigation, it was concluded that the pigment production produce throughout the first 24 hrs of growth and maximal pigment production was reached following 48 hrs. While, isolate No. 4 achieved the highest yield after 72 hrs. Among these examined strains, the characteristic of Pigment production was found in the 4 (26.6 %) out of 15 strain had this ability to produce pigment vigorously within 48 h of incubation as in figure (3).

**Figure 3. Growth of *P. aeruginosa* on the tested media with produce pigment**

Pyocyanin that generated by *P. aeruginosa* is employed in the various clinical microbiological laboratories as an adjunct test in the multiple testing procedures adopted for the identification of *P. aeruginosa*. In preceding researches, the pyocyanin production and catalase activity were enhanced when *P. aeruginosa* was grown in low- and high-phosphate succinate media under conditions of limited Phosphate (10).

**Extraction and chemical analysis of pigment**

In the current investigation, a chloroform solvent was the addition for departed of pyocyanin from culture supernatants Chloroform extracted layer of pyocyanin showed converter in color from bluish to pinkish red during acidified by 0.1 (N) HCl, which indicated the presence of pyocyanin pigment. Chloroform extracted of *P. aeruginosa* revealed on gas chromatographic analysis there is a sharp peak at acquisition period 27.13 minutes that recognized as (Hemipyocyanin) alpha-Hydroxy phenazine through mass spectrum analysis which provided intense molecular ion peak at 205 m/z and its structure is presented in Figure(4).

**Figure 4. Illustrated the mass spectrum analysis of pyocyanin**
GC-Ms of pyocyanin in the current investigation revealed the existence of phenazine and Hemipyocyanin compound. Prior analysis of GC-Ms by (14) confirmed these result that revealed the correlated hemipyocyanin pigment extracted of *P. aerogenosa* which recognized by mass spectrum following gas chromatography at ions peak (211 m/z) while the estimated one is 211.09 for C13H11N2O. And also consonant with the previous studies of (2) who demonstrated a molecular ion of the protonated purified compound of pyocyanin at m/z 196.

**Antimicrobial activity against the target bacteria**

The antimicrobial activity of purified pyocyanin at different concentrations (25, 50, 75 and 100 mg/ml) was observed towards Gram-negative and Gram-positive bacteria that isolated out of minced meat. One strain was chosen for the production of (pyocyanin) and estimated for its antibacterial activity by agar well diffusion technique. Out of various concentrations of pigments that used, 25 mg/ml revealed less activity with moderate inhibition zone on the agar plate. The remaining concentration of 50-75 mg/ml revealed the significant obligation with a higher zone of inhibitory activity, while the 100mg/ml which is considered the higher concentration and high purity recorded the higher inhibition zone compared with (25, 50 and 75 mg/ml) and the results are presented in table (3) and figure (5).

**Table 3. Pyocyanin concentration of *P.aeruginosa* with Diameter of inhibition zone on target Bacteria**

| Bacterial isolates         | Pyocyanin concentration of *P.aeruginosa* with Diameter of inhibition zone on target Bacteria (mm) | LSD value |
|---------------------------|-------------------------------------------------------------------------------------------------|-----------|
|                           | 25% | 50% | 75% | 100% |                            |           |
| *Psychromonas fluorescens*| 0   | 2   | 4   | 7    | 3.63 *                        |           |
| *Escherichia coli*        | 12  | 18  | 22  | 27   | 5.92 *                        |           |
| *Klebsiella sp*           | 8   | 11  | 17  | 26   | 5.77 *                        |           |
| *Staphylococcus aureus*   | 13  | 17  | 22  | 29   | 5.08 *                        |           |
| *Bacillus cereus*         | 4   | 7   | 11  | 14   | 4.42 *                        |           |
| *Proteus mirabilis*       | 3   | 6   | 8   | 13   | 4.68 *                        |           |
| *Proteus vulgaris*        | 4   | 6   | 9   | 11   | 4.09 *                        |           |
| *Serratia marcescens*     | 7   | 14  | 19  | 24   | 6.16 *                        |           |
| *Enterobacter sp*         | 5   | 8   | 13  | 17   | 4.51 *                        |           |
| *LSD value*               | 4.78 * | 5.02 * | 5.64 * | 6.83 * | ---- |

* (P<0.05).

The common influenced bacteria to pyocyanin was *E. coli* and *staph.aureus* followed by *Serratia marcescens* and *Klebsiella sp*. at the same level. While *Enterobacter sp, Bacillus cereus, Proteus mirabilis, and Proteus vulgaris* showed intermediate sensitivity, the *Psychromonas fluorescens* was shown a weak low sensitivity to pyocyanin. These conclusions are in accordance with (10) they notify that phenazine compound has antimicrobial activity entirely toward *Bacillus subtilis* strains and *Escherichia Coli*. There was a considerable variation of the results regarding the bacterial resistance obtained from different strains and association with pyocyanin of isolated bacteria, This variation refers to the lipid of the cell wall content of Gram-negative and Gram-positive bacteria that may be accountable to the difference for the sensitivity of the pyocyanin antibiotic. Through expanding pyocyanin concentration from 50 mg/ml to 100 mg/ml, the antimicrobial activity is improved and enhanced; therefore the pyocyanin is concentration dependent as an antibiotic activity. Pyocyanin exhibits as a redox cycle and enhances intracellular oxidant stress and within the aerobic situation. This drives to reactive oxygen species (ROS) generation like hydrogen peroxide, and superoxide, these ROS compounds are able to inhibit the growth of microorganism (8).
The current investigation concluded that the pyocyanin which extracted from *Pseudomonas aeruginosa* isolated out of minced meat was hemipyocyanin and has antimicrobial function as competitive agents infectious and pathogenic bacteria which contaminated food and these could assist as a signal to alert *P. aeruginosa* to the presence of another bacteria and the consequent progressed in pyocyanin production would help *P. aeruginosa* to compete with these microbes and save the food from contamination with pathogenic bacteria.

**REFERENCES**

1. Abd El-Aziz, D.M 2015. Detection of *Pseudomonas spp.* in chicken and fish sold in markets of assiut city, Egypt. Journal of Food Quality and Hazards Control, 2: 86-89
2. Abdul-Hussein, Z. R; and S.S., Atia 2016. Antimicrobial effect of pyocyanin extracted from *Pseudomonas aeroginosa*. European Journal of Experimental Biology, 6 (3):1-4
3. Alasalvar, C.; F., Shahidi; K., Miyashita; and U., Wanasundara 2011. Handbook of seafood quality, safety and health applications. Blackwell Publishing Ltd, UK
4. Altaai, M.E.; I.H.,Aziz; and A.A., Marhoon 2014. Identification *Pseudomonas aeruginosa* by 16s rRNA gene for Differentiation from Other *Pseudomonas Species* that isolated from Patients and environment. Bagh. Sci. Jou, 11 (2): 1028-1034
5. Benie, C.K.; A., Dadié; and A., Guesennd 2017. Characterization of virulence potential of *Pseudomonas aeruginosa* isolated from bovine meat, fresh fish, and smoked fish. European Journal of Microbiology and Immunology, 7(1):1–10
6. CA-SFM/EUCAST. 2015. European committee on antimicrobial susceptibility testing and antibiogram. Committee of the French Society of Microbiology, 158
7. Cheluvappa, R 2014. Standardized chemical synthesis of *Pseudomonas aeruginosa* pyocyanin. MethodsX, (1): 67–73
8. Das, T; and M., Manefield 2012. Pyocyanin promotes extracellular DNA release in *Pseudomonas aeruginosa*. PLoS ONE, 7(4): 67-18
9. El-Fouly, M.Z.; A.M., Sharaf; A.A.M., Shahin; A. Heba; and A.M.A., El-Bialy 2015. Biosynthesis of pyocyanin pigment by *Pseudomonas aeruginosa*, Journal of Radiation Research and Applied Sciences, 8: 36–48

10. El-Shouny, W.A.; A.R.H., Al-Baidani; and W.T., Hamza 2011. Antimicrobial Activity of Pyocyanin Produced by *Pseudomonas aeruginosa* isolated from Surgical Wound-Infections. *International Journal of Pharmaceutical and Medicinal Research*, 1(1): 01-07

11. Gholami, A.; A., Majidpour; and M., Talebi–taher 2016. PCR–based assay for the rapid and precise distinction of *Pseudomonas aeruginosa* from other *Pseudomonas* species recovered from burns patients. Journal of preventive medicine and hygiene, 57(2):81–85

12. Hassan, I.H.; S.A., Rafik; and K., Mussum 2012. Molecular identification of *Pseudomonas aeruginosa* isolated from Hospitals in Kurdistan region. Journal of Advanced Medical Research, 2(3): 90-98

13. Jay, J.M.; M.J., Loessner; and D.A., Golden 2005. Modern food microbiology. 7th edition. Springer Science, USA

14. Priyaja, A. 2013. Pyocyanin (5-Methyl-1 Hydroxyphenazine) Produced by *Pseudomonas aeruginosa* as Antagonist to Vibrios in Aquaculture: Overexpression, Downstream Process and Toxicity (Ph.D. thesis) Cochin Univ. of Science and Technology, India

15. Priyaja, P.; P., Jayesh.; N, Correya., B, Sreelakshmi., N, Sudheer; and R 2014. Antagonistic effect of *Pseudomonas aeruginosa* isolates from various ecological niches on Vibrio species pathogenic to crustaceans. Journal of Coastal Life Medicine, 2, 76e84

16. Quinn, P.J.; B.K., Markey; F.C., Leonard; P., Hartigan; S., Fanning; and P.E.S., Fitz2011.Veterinary microbiology and Microbial Disease. 2nd ed. UK: Wiley – Blackwell West Sussex, 978(1):118-302

17. Sambrook, J; and D., Russell 2001. Molecular cloning: a laboratory manual 3rd. cold Spring Harbork, New York: cold spring laboratory

18. SAS. 2012. Statistical Analysis System, User's Guide. Statistical. Version 9.1th ed. SAS. Inst. Inc. Cary. N.C. USA

19. Sudhakar, T.; S., Karpagam; and S., Shiyama 2013. Analysis of pyocyanin compound and its antagonistic activity against phytopathogens, *International Journal of Chemistry Research*, 5: 1101–1106

20. Virupakshiaiah, D.B.M; and V.B., Hemalata 2016. Molecular identification of *Pseudomonas aeruginosa* from food borne isolates. *International journal of current microbiology and applied science*, 5(6):1026–1032.