Enhancement of pyocyanin production by *Pseudomonas aeruginosa* using biotic and abiotic factors

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Abstract

Due to the importance of pyocyanine pigment as an antimicrobial agent, the present study was designed to induce the production of this pigment from Pseudomonas aeruginosa using biotic and abiotic factors. Different culture media were used for production of this blue pigment, among them, louria broth LB was found the best production medium in which the concentration of pyocyanine reached to 21.5µg/ml by using this medium. Three bacterial cells were used as biotic factors for enhancing the production of pyocyanine, the results showed the ability of these bacteria for enhancement and the best elecitor concentration was 500 and 750 µl for Staphyllococcus aureus and Klebsiella pneumonia, 250 and 500 µl for bacteria B. subtilis.

Different types of oils were used in this study as abiotic elicitors, all used oils lead to increase the production, but the maximum increase obtained with the use of olive and petroleum oils. Two types of nanoparticals in different concentrations were used in this study as abiotic elicitors, the result demonstrated that the used nanobarticals had the ability of enhancing the most productive of the blue pigment and three concentrations lead to increasing the production (5, 10, 20 mg/ml) in the case of ZnO_2 nanobartical, but the best enhancing concentration for Fe_3O_2 was 5 and 10 mg/ml. The results revealed that pyocyanine had antibacterial activity against all gram positive and negative tested pathogenic bacteria.

Keywords: *Pseudomonas aeruginosa*, Pyocyanin, Nanoparticals, Edible oils, *Klebsiella pneumoniae*.

Introduction

It is known that bacteria produce wide range of pigments for several purposes, one of these bacteria is *Pseudomonas aeruginosa*, which is a gram-negative, rod shaped. It is widely spread in different environments such as soil, water, humans, animals, plants, sewage and hospitals[1] that produce pyocyanin, one of the extra-cellular pigment from a group of nitrogen containing heterocyclic bioactive compound. Pyocyanin is a water-soluble blue, green pigment and has antibiotic activity against different microorganisms.[2,3] It was recognized as an organic base, blue in alkaline solutions, but converted to red color when acidified, its chemical reduction leads to a colorless form, spontaneously oxidized in air.[4] At the beginning pyocyanin had been used as a reversible dye with a redoxpotential similar to that of menaquinone.

Pyocyanin has different pharmacological impacts on prokaryotic cells, its biological activity is related to similarity in the chemical structure to Isoalloxazine, flavoproteins, flavin mononucleotide and flavin adenine dinucleotide compounds.[5] Pyocyanin also has antibiotic activity toward different microorganisms and the antibacterial activity of pyocyanin against different bacteria such as *Staphylococci* and *Vibrio* sp. The main aim of the present work was to study of the effect of different biotic and abiotic elicitors in enhancing the production of pyocyanine pigment by locally isolate of *Pseudomonas aeruginosa.*

Materials and Methods

Microorganisms: Locally isolated *Pseudomonas aerouginosa* produces pyocyanine pigment. Pathogenic bacteria used in the present study as inducers and for antibacterial activity test of pyocyanine pigment (*Staphylococcus aureus, Klebsiella pneumonia, Bacillus subtilis* and *E. coli*), bacteria were obtained from Department of Biotechnology/ College of Science/ University of Baghdad.

Production of pyocyanine by obtaining *Pseudomonas* isolate: *Pseudomonas aeruginosa* was previously isolated from hydrocarbon soil and recognized using gram staining and standard biochemical test as well as molecular identification[8] and was conserved in Pseudomonas agar slants and kept at 4ºC in refrigerator. A loopful from the surface of Pseudomonas agar containing this isolate was added to flask containing 50 ml of Peudomonas broth medium (pH = 7) in duplicate, then the flasks were incubated for 2-3 days, 121 rpm at 30ºC in a shaker incubator. The variation in color of the culture of bluish green sign posted the pigment production. 5 ml of sample was centrifuged at 10000 rpm for 10 min and 3ml of chloroform was added to the supernatant and shake well until blue color is obtained. It was further confirmed by adding 0.2 N HCl to the blue color pigment and the color must turn to red.[9]

The effect of using different media components on production of pigment: Five culture media (Louria Broth LB, Pseudomonas Broth PB, Cetramide Broth CB, Mineral Salt Broth MSM and Nutrient Broth NB) were used for the production of pyocyanin pigment. Erlenmeyer flasks (250ml) having 50ml of each tested medium (pH = 7) in duplicates were sterilized, then inoculated with 1 ml of broth containing *Pseudomons* isolate. Flasks were put on a rotating shaker (120 rpm) for 72 h at 30 ºC. After the incubation, each

flask was centrifuged at 10000 rpm for 10 minutes. Lastly the supernatant was transported to a cuvette for the measurement of absorbance at 520 nm and then, the concentration of produced pigment was calculated.

Biotic Enhancement in the production of pyocyanine pigment: Live cells of the bacteria *Staphylococcus aureus*, *Klebsiella pneumonia* and *Bacillus subtilis* were used in the present study as biotic elicitors for the production of pyocyanine pigment by *Pseudomonas* bacterium. Inoculum of these bacteria was equipped as follows: Loopful of every bacterial growing at overnight culture on nutrient agar were inoculated into 250 ml Erlenmeyer flasks containing 50 ml of LB broth (pH= 7) and incubated at 37°C for 24 hours. After the incubation, an McFarland standard tube was used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria cells adjusted to be nearly 1×10^8 cells/ml by adding fresh sterile normal saline if necessary. Then, the inoculum of each elicitor was centrifuged at 10000 rpm for 15 min. Bacterial cells were then washed and re-suspended by adding 15 ml of sterile normal saline.

The number of *Pseudomonas* cells were adjusted to 1×10^8 cells/ml by using the same strategy as mentioned above. Then LB medium was inoculated with prepared *Pseudomonas* inoculum at a level of 1% (v/v). At zero-time, elicitor inoculums were added to the flasks that containing *Pseudomonas aeruginosa* separately at a level of 250 µl, 500 µl, 750 µl and 1000 µl, one flask containing 50 ml LB medium was inoculated with 1ml of *Pseudomonas* inoculum used as a control in this experiment. Then flasks were incubated at 30 C° for 72 h. Tasters were taken at Zero time, 24h, 48 h and 72 h throughout the culture for the measurement of the absorbance at 520nm and calculation of pigment concentrations.

Biotic Enhancement by using various oil sources: To determine the effect of using different oils as inducers in the production of pyocyanine pigment, 1 ml of various oils (Soybean oil, Castor oil, Olive oil, Black seed oil and petroleum oil) was added in duplicate to ten (250ml) conical flasks, everyone containing 50 ml of LB medium at pH 7. After sterilization, the flasks were inoculated with 1ml of broth containing *Pseudomonas* isolate and incubated in a rotary shaker (120 rpm) at 30ºC for 72 hours. One flask containing sterile LB medium at pH 7 was inoculated with *Pseudomonas* also used as control. After the incubation, supernatant was taken for the determination of absorbance at 520 nm and calculation of pigment concentrations.

Biotic Enhancement by using different concentrations of ZnO_2 and Fe_3O_2 nanoparticles: Different concentrations (5, 10, 20, 30 and 40 mg/ml) of ZnO_2 and Fe_3O_2 nanoparticle were prepared using deionized distilled water and Sonication technique. One ml of each concentration was added separately to ten flasks containing sterile LB medium at pH 7 inoculated with 1ml of broth containing *Pseudomonas* inoculum, then the flasks were incubated in a rotating shaker (120 rpm) at 30ºC for 72 hour. After the incubation, supernatant was taken for the determination of absorbance at 520 nm and determination of pigment concentrations.

The concentration of pigment production in each experiment of the present study: In each experiment above, after incubation, the culture media from each flask was taken and centrifuged at 10000 rpm for 10 min, then the absorption was measured at 520 nm for the blue colored supernatant. The produced pigment concentrations, expressed as micrograms of pyocyanin produced per ml of culture supernatant, were also calculated using the following equation:[10]

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

Tested the antibacterial activity of pyocyanine: The Nutrient broth was inoculated with pathogenic bacteria *E. coli*, *S. aureus*, *B. subtilis* and *K. pneumonia* incubated overnight at 37°C. Formerly 100 µl of each bacterial suspension was dispensed on the surface of Muller Hinton Agar spread by L- shape glass rod and left for 10 minutes to calm down the bacteria and 100 µl of supernatant containing pyocyanin pigment was added to the prepared wells in the same plate and incubated at 37°C for 24 hrs., the diameter of the zones was measured and the results were documented.

Results and Discussion

Production of pyocyanine by *Pseudomonas* isolate: Production of pigment by *Pseudomonas* isolate was accomplished after 72 hours incubation. Soluble pigment called pyocyanin was sign posted by a change in the color of solid media in which pyocyanin production was demonstrated as green color (figure 1). Blue- green pigment was obtained from *Pseudomonas* isolate grown in liquid medium (fig. 2). Production of this pigment was also confirmed by an alteration in the color of the pigment from deep pink to red upon addition of chloroform and acidified using 0.2 (N) HCl (fig. 3).[11,12]

Fig. 1: Pyocyanine pigment produced on solid medium

Fig. 2: Blue green pigment in liquid medium

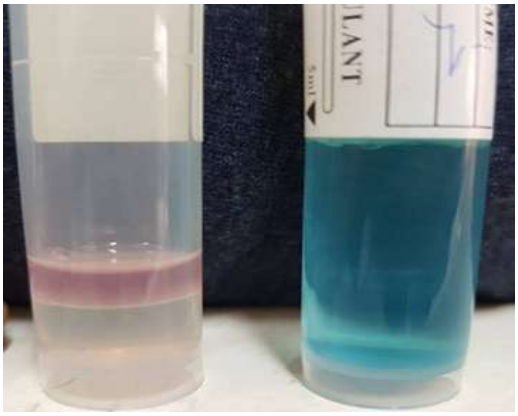

Fig. 3: Bluish extract of pyocyanin converted to deep pink when acidifying with HCl

The effect of using different media components on production of pyocyannine: The influence of culture media on the production of pyocyanine pigment was tested by cultivating the isolate *Pseudomonas aeruginosa* in five different media. The results showed the ability of using isolate for producing this blue- green pigment in all tested media, but louria broth was much better support medium for producing of pyocyanine. Concentration reached to 21. 8 µg/ml in comparison with other culture media (MSM= 12 µg/ml, NB= 8 µg/ml, CB= 15 µg/ml and PB 18.5 µg/ml) as shown in fig. 4. Therefore, this medium was selected to complete the other experiments in the present study.

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The results are in agreement with the study of Sudhakar and his work group[12] who reported that *Pseudomonas aeruginosa* SU1 isolated from several samples have the ability to produce pyocyanine pigment in various media and with the study of Young[13] who conveyed that the bacteria *Pseudomonas aeruginosa* had the ability to produce pyocyanine pigment in different media.

Enhancement by using live cells of the bacteria *Staphylococcus aureaus, Klebsiella pneumonia* and *Bacillus subtilis*: The addition of live cells of *S. aureaus, K. pneumonia and B. subtilis* to *P. aeruginosa* culture had a great influence on the creation of pyocyanine as their concentrations were significantly increased. Several concentrations of the elicitor cells were tested. The results showed that concentrations 500 and 750 µl in the case of *S.aureaus* and *Klebsiella pneumonia* had a great effect as the concentrations of producing pigment were maximum increased on the day three of incubation in comparison with the pure culture (control) as shown in fig. 5 and 6.

But with the addition of *B. subtilis*, the concentrations (250 and 500 µl) affect the growth of *Pseudomonas* isolate and lead to increase in pigment production as demonstrated in fig. 7.

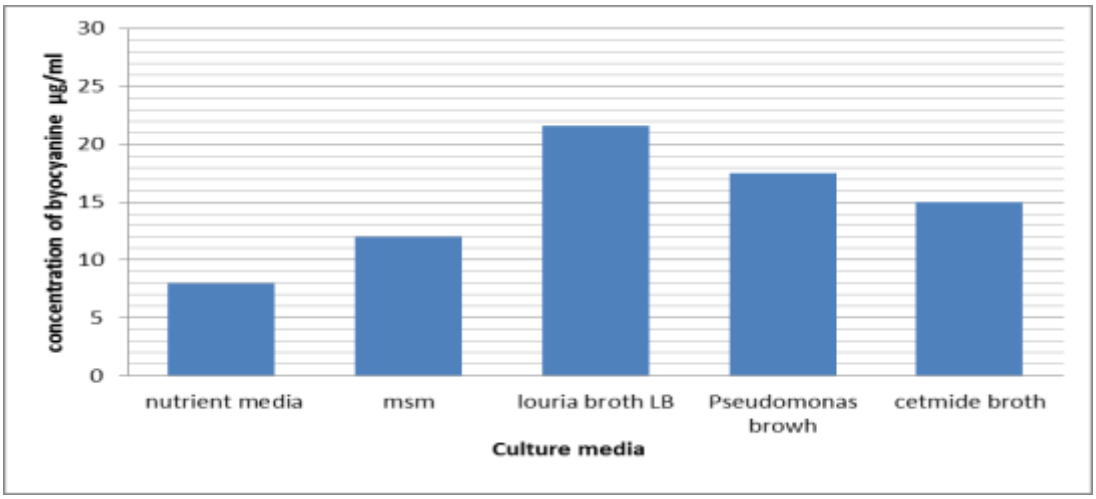

Fig. 4: Production of pyocyanine by *Pseudomonas aeruginosa* in diverse culture media

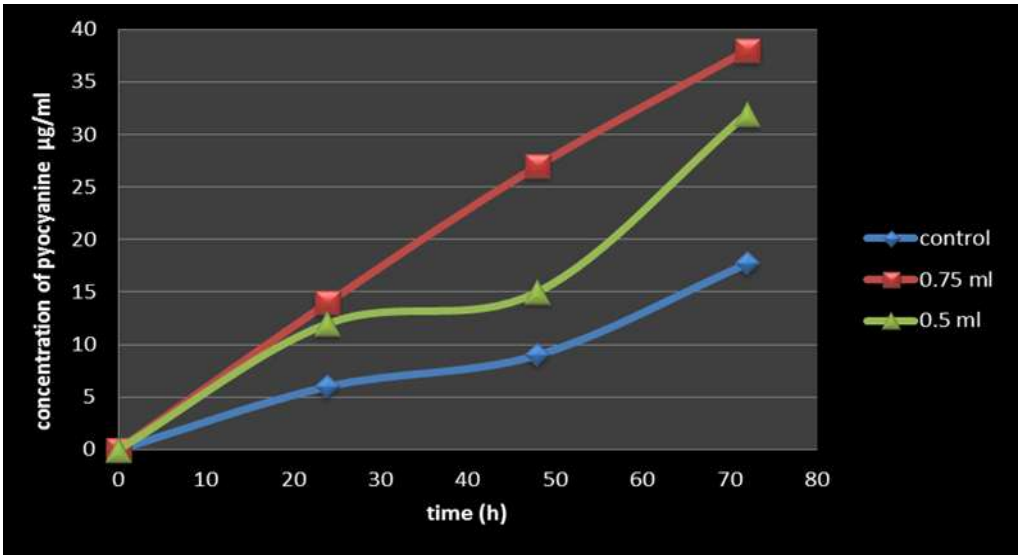

Fig. 5: Pyocyanine produced by *Pseudomonas aeruginosa* enhancing with live cells of *S. aureaus*.

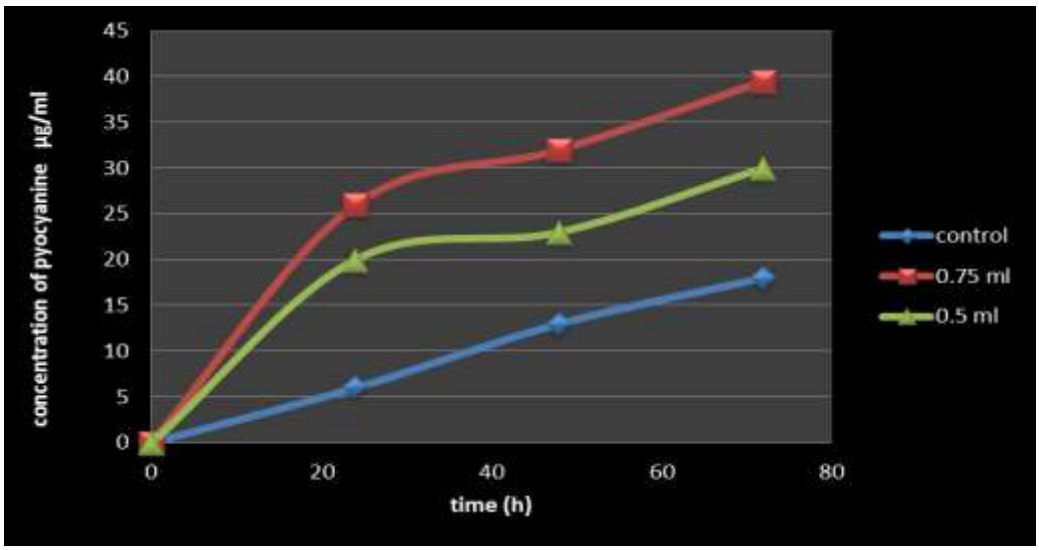

Fig. 6: Pyocyanine produced by *Pseudomonas aeruginosa* enhancing with live cells of *K. pneumonia.*

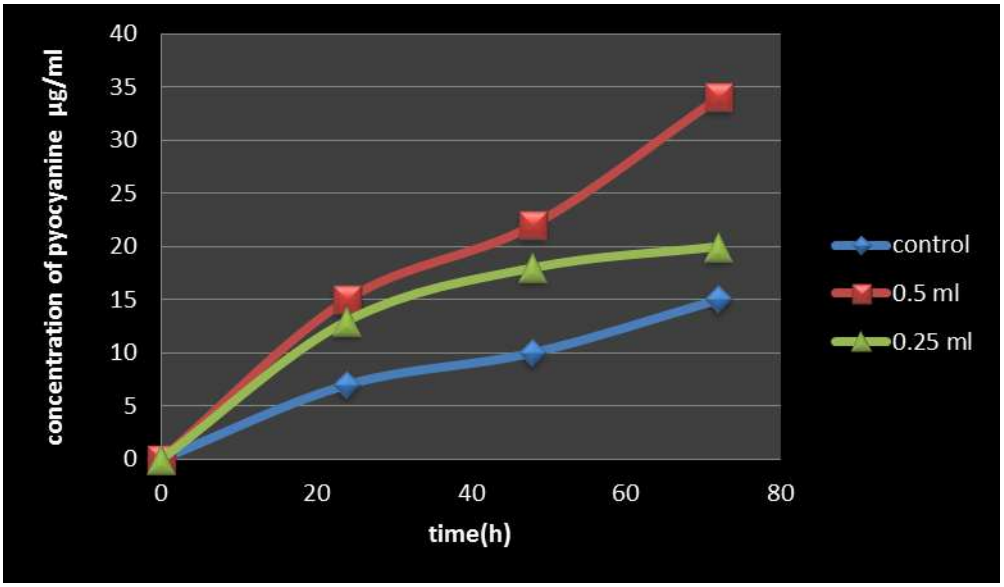

Fig. 7: Pyocyanine produced by *Pseudomonas aeruginosa* enhancing with live cells of *B. subtilis*.

These results were compatible with the results obtained by Khalid and Reem[14] who found that the addition of live and destroyed cells of *E. coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae* enhanced phenazine pigment production by the bacteria *Pseudomonas aeruginosa.*Also, Samer and his work group[15] reported that introducing microbial elicitor cells to *Serratia marcescens* culture media led to enhance the production of prodigiosin pigment.

To enhance the production of pyocyanine by using abiotic factors

Enhancing by using various oils: Louria broth media containing edible and heavy oils were used for enhancing the production of pyocyanine from *Pseudomonas aeruginosa*. The edible oils and petroleum oil were found to be more suitable for pyocyanine production. Out of the different oils used, olive oil and petroleum oil were found the best sources for enhancing the production of this pigment which lead to increasing their concentration of 33 µg/ml and 35 µg/ml, respectively in comparison with the concentration of pyocyanine in pure culture (control) which was 15µg/ml and the other oils used (Black seed oil = 22, Castor oil = 26.5 and Soybean oil = 30µg/ml) as shown in fig. 8.

Many studies showed that addition of oils to culture media supports the production of microbial secondary metabolites. Shahitha and Poornima[16] reported that addition of different oil sources in culture medium enhanced the production of prodigiosin pigment in *Serratia Marcescens*. Swaadoun et al[17] revealed that addition of different oil sources to cultures of *Streptomyces*sp. 6621 enhanced the production of Cephamycin C antibiotic by this bacteria.[17]

Enhancing by using different concentrations of ZnO_2 and Fe_3O_2 Nanoparticles: Different concentrations of ZnO_2 and Fe_3O_2 nanoparticles (5, 10, 20, 30 and 40 mg/ml) were added to *Pseudomonas aeruginosa* cultures for increasing the production of pyocyanine pigment from this bacterial isolate. The results demonstrate that three concentrations of ZnO_2 nanoparticle (5, 10 and 20 mg/ml) had a important effect on increasing the production of this pigment as the their concentrations were increased to 33, 30 and 28 µg/ml respectively in comparison with the culture of *Pseudomonas aeruginosa* free from nanoparticle which was 19 µg/ml, while the 30 and 40 mg/ ml concentrations of ZnO_2 nanoparticle had a negative effect on the pigment production as shown in fig 9.

In the case of Fe_3O_2 nanoparticle, two concentrations (5 and 10 mg/ml) lead to increasing the production of this pigment as the their concentration were increased to 35 and 31 µg/ml respectively in comparison with control culture which was 16 µg/ml, while the concentrations 20, 30 and 40 mg/ ml of this nanoparticle had a negative effect on the pigment production as shown in fig. 10.

There are no previous studies documented using the nanoparticles as elicitors for enhancing the production of secondary metabolites by microorganisms.

Antibacterial activity of pyocyanine pigment: *Pseudomonas aeruginosa* are known for synthesizing different bioactive compounds, among these pyocyanin is best known for its antimicrobial action against different bacteria such as *Clostridium botulinum*, *S. aureus*, B. *subtilis*, *S. epidermis*, *Micrococcus luteus*, in addition to *B. licheniformis*.[18-20] In the current study the antibacterial activity of pyocyanine was described against *E. coli*, *S. aureus*, *B. subtilis* and *K. pneumonia*. It can be observed from the table 1 and fig. 11, that pyocyanine had good antimicrobial activity against all tested gram positive and gram negative pathogenic bacteria with inhibition zone reaching to 38 mm against bacteria *E.coli.*

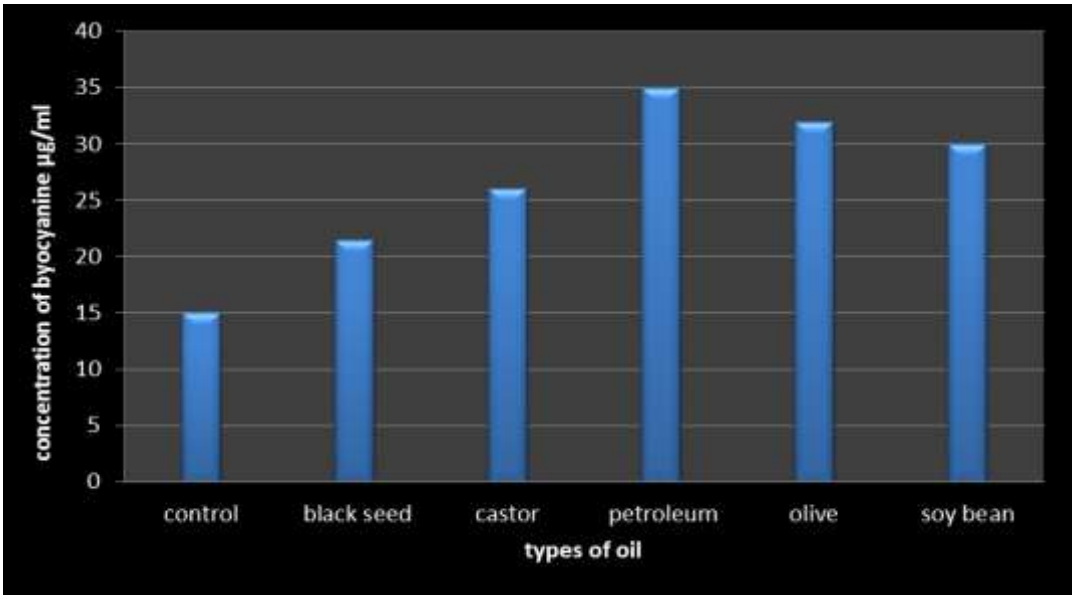

Fig. 8: Enhancing the production of pyocyanine pigment by *Pseudomonas aeruginosa* using different oils.

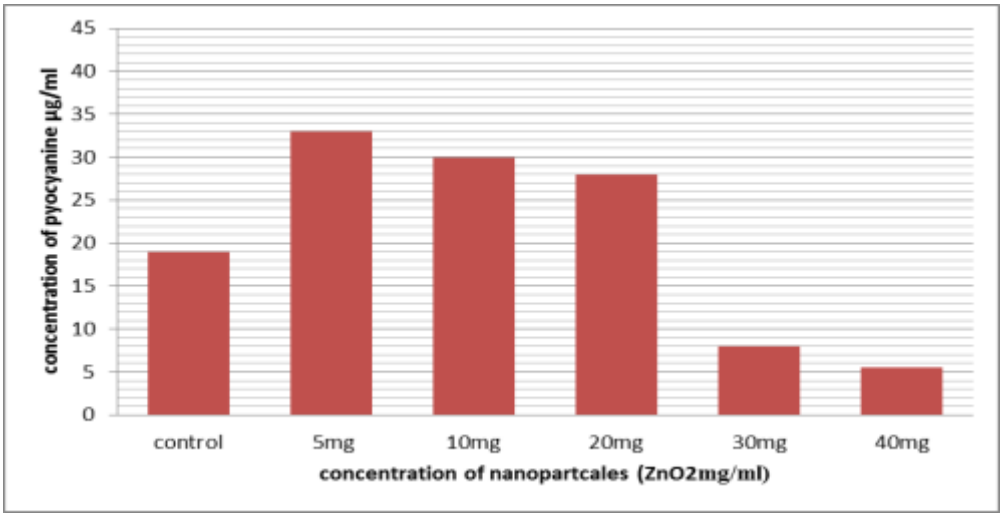

Fig. 9: Pyocyanine pigment created by *Pseudomonas aeruginosa* using diverse concentrations of ZnO_2 nanoparticle.

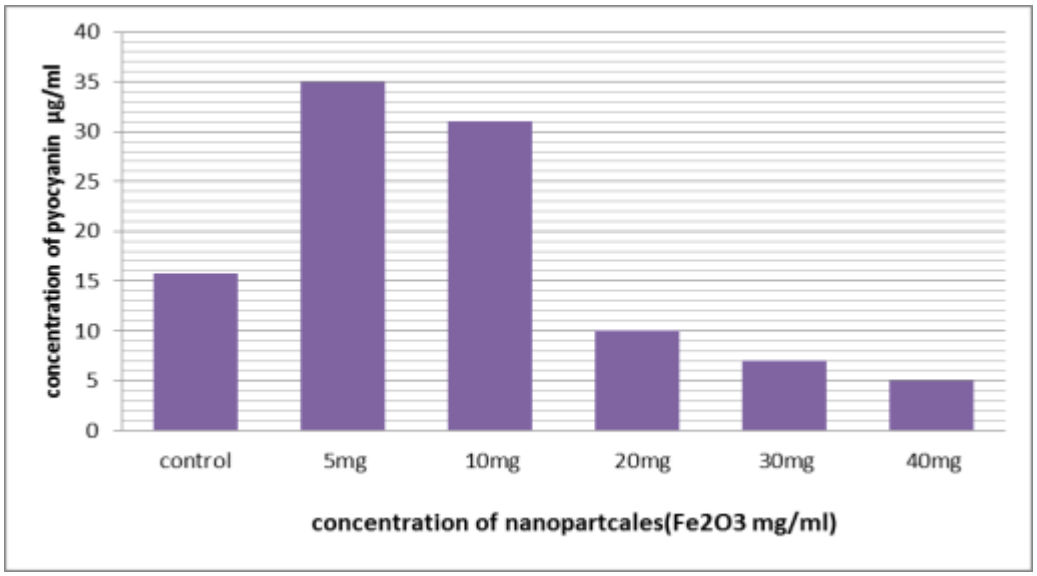

Fig. 10: Pyocyanine created by *Pseudomonas aeruginosa* using diverse concentrations of Fe_3O_2 nanoparticle.

Fig. 11: Antibacterial action of pyocyanine pigment formed by *Pseudomonas aeruginosa*

Table 1
The antibacterial activity of pyocyanine pigment

Pathogenic bacteria	Diameter of inhibition zones(mm)
E. coli	38
S. aureus	34.5
B. subtilis	26
K. pneumonia	28

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