EVALUATION THE ABILITY OF TITANUM OXIDE NANOPARTICLES TO INCREASE THE PRODUCTION OF PRODIGIOSIN AND PHENASINE FROM *Serratia marcescens* AND *Pseudomonas aeruginosa* RESPECTIVELY

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ABSTRACT

The present study was aimed to investigate the possibility of titanium oxide nanoparticles to enhance the production of both prodigiosin by *Serratia marcescens* and phenazine by *Pseudomonas aeruginosa.* Moreover; the poor non-selective nutrient broth was used instead of using the selective nutrient broth for the production of both compounds to reduce the economic cost for production. Different concentrations of titanium oxide nanoparticles (0.005, 0.01, 0.015 mg/L) were used in this study to choose the most suitable concentrations to increase production. Both prodigiosin and phenazine were considered promising drugs for treating many diseases owing to their characteristics of antibacterial, antifungal, immunosuppressive, and anticancer activities. The results revealed that both the prodigiosin and phenazine production was increased from *S. marcescens* and *Pseudomonas aeruginos*a when using titanium oxide nanoparticles at (0.01 mg/L) concentration and the size of an average diameter was 57.07 nm.

Keywords: TiO_2 NPs, *P. aeruginos*a, *S. marcescens*, growth inhibition, antibacterial.

مجلة العلوم الزراعية العراقية -2022 :53(3):496-504 يعقوب وآخرون

تقييم قابلية جزيئات أوكسيد التيتانيوم النانوية لزيادة انتاج prodigiosin و phenazine من بكتريا *Serratia marcescens* و *Pseudomonas aeruginosa* على التوالي

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المستخلص

هدفت الدراسة الحاليه الى التحري عن امكانية استعمال جزيئات أوكسيد التيتانيوم النانوية (TiO_2 NPs) في زيادة انتاج كل من المضادين الحيويين الـ (prodigiosin) من بكتريا *Serratia marcescens* والـ (phenazine) من بكتريا *Pseudomonas aeruginosa* بالاضافه الى ذلك تم الاعتماد على استخدام مرق مغذي فقير غير انتخابي بدلا من المرق المغذي الانتخابي الخاص بانتاج كلا المركبين من اجل تقليل الكلفه الاقتصاديه للانتاج. تم استخدام تراكيز مختلفة من جزيئات أكسيد التيتانيوم النانوية (0.005 ، 0.01 ، 0.015 ملغم / لتر) في هذه الدراسة لاختيار التركيز الأنسب لزيادة الإنتاج. يعتبر كل من (prodigiosin) و (phenazine) دواءً واعدًا لعلاج العديد من الأمراض نظرًا لخصائصها المتمثلة في الأنشطة المضادة للبكتيريا والفطريات والمناعة والمضادة للسرطان. لذا كان لابد من البحث عن طرق حديثه لزيادة انتاج كلا المركبين لاهميتهما خصوصا في المجال الطبي. أظهرت النتائج زيادة في انتاج كل من (prodigiosin) و (phenazine) بعد استعمال جزيئات أوكسيد التيتانيوم النانوية بتركيز (0.01 ملغم /لتر) وبحجم 57.07nm .

الكلمات المفتاحية: جزيئات التيتانيوم النانوية، *P. aeruginosa*، *S. marcescens* ، تثبيط النمو، مضاد للبكتريا.

Received:11/2/2021, Accepted:15/4/2021

INTRODUCTION

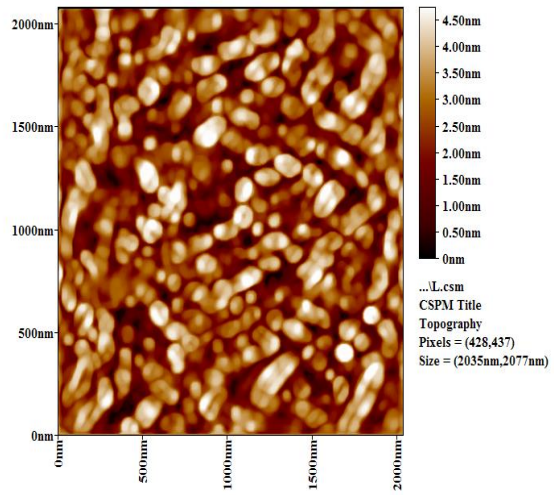
Among the most important discoveries of medical science are Antibiotics. Analysis of infectious disease mortality data from the U.S. government reveals that antibacterial agents may save over 200,000 American lives annually (2,14). Antibiotics usually synthesized via many different enzymatic steps through several complex pathways which are normally cost energy. However, the question is, if they are not essential; why do microorganisms produce them? Unfortunately, there is no complete and clear answer yet. They must be important to the habitat. Though, a massive development has been made towards finding and understanding the biosynthesis, mode, and mechanisms of their actions (22). Secondary metabolites produced by bacteria are relatively small molecules, each produced by a limited number of strains that appear to have no function in the growth. In fact, by mutation producer strains have lost their production ability to exhibit perfectly normal growth rates and characteristics. Antibiotics, antitumor agents, pigments, toxins, immunomodulating agents, pheromones, enzyme inhibitors, receptor antagonists and agonists, pesticides, and growth promoters of animals and plants are secondary metabolites (28). Microorganisms in the natural habitat normally produce antibiotics in low concentrations (15). *S. marcescens* is a species of the gram-negative bacillus of the *Enterobacteriaceae* (13, 20, 27). In the hospital environment this microorganism is widely distributed and as an opportunistic pathogen has been recognized and causing a variety of nosocomial infections, including wound infections, skin, and soft tissue infections and many others infections (29). In recent years, *S. marcescens* was used for the study of secondary metabolites as a model organism (19). However, as secondary metabolites, prodigiosin is a tripyrrole red-colored compound produced by many terrestrial (soil) and marine bacterial strains including species of *Serratia* (26). This compound has been spurred by its antitumor, immunosuppressant, and antimalarial activities at nontoxic levels (33). *Pseudomonas aeruginosa* is a Gram-negative opportunistic bacterium that inhabits the soil and surfaces in aqueous environments normally. Its adaptability and high intrinsic antibiotic resistance enable it to survive in a wide range of other natural and artificial settings, including surfaces in medical facilities. From the organism's ability to produce exotoxins harmful to humans, *P. aeruginosa* pathogenicity and survival were produced and regulated in part by cell-to-cell interaction (24, 30). The production of many bactericidal and fungicidal compounds synthesized by *P. aeruginosa* was regulating, thus the bacteria's action of defense was modulating (15, 20). Antibiotics isolated from the extracts of *P. aeruginosa* culture supernatant included pyoluteorin, phenazine-1-carboxamide, and five members of the 4-hydroxy-2-alkyl / alkenyl quinolines. Phenazines are a class of tricyclic aromatic molecules produced by P. aeruginosa and several other Gram-negative and Gram-positive bacteria (30). Some phenazines, pyocyanin especially, have been shown to act as toxins against bacteria or mammals fungi as a consequence of their redox activities (16). The science that deals with the processes that occur at the molecular level and of Nano length scale size are Nanotechnology, which involves the tailoring of materials at the atomic level to attain unique properties, which for desired applications can be suitably manipulated (20). By controlling the shape and size of the nanoscale nanotechnology can also be defined as the design, production, characterization, and application of materials, systems, and devices (3). Nanotechnology is helping to considerably improve many technologies and industry sectors. Among applications of nanotechnology in the different fields are in medicine (drug delivery, medical devices, sensing, tissue engineering), food sciences (processing, nanocapsules), chemicals and cosmetics (nanoscale chemicals and compounds, paints, coatings), environment and energy (water and air purification food sciences (processing, nanocapsules), photovoltaic), food sciences (processing, nanocapsules), military and energy (biosensors, sensory enhancement), electronics (semiconductors chips, memory storage, photonics, optoelectronics) and scientific tools (atomic force, microscopic and scanning

tunneling microscope) (4, 31). The naturally occurring oxide of titanium, Titanium dioxide, which is also known as titania, has the chemical formula (TiO_2). Generally, it is sourced from ilmenite, rutile, and anatase. It has a wide range of applications, from paint to sunscreen to food coloring. When used as a food coloring, it has E number E17. Also, researchers recorded its antibacterial and antitumor activities (5). Depending on what is known about the importance of both titanium oxide and nanotechnology, this research is aimed to synthesize titanium oxide nanoparticles and study it is the ability to enhance or increase the production of secondary metabolites by bacterial strains.

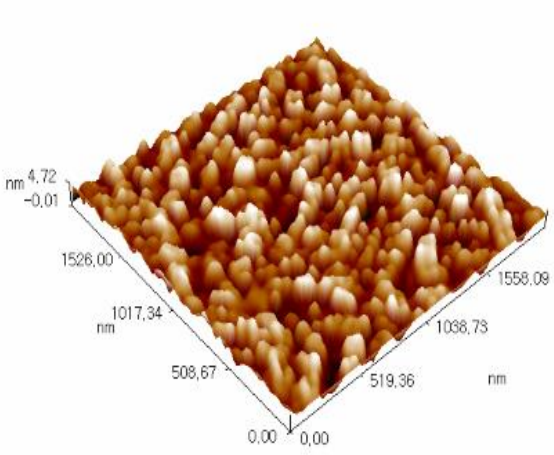
MATERIALS AND METHODS

Synthesis of TiO_2 nanoparticles and characterization: All the chemicals and reagents used for the preparation of TiO_2 nanoparticles were purchased from (Sigma, Ltd). The leaves of Aloe Vera were separated from the plant, which was thoroughly washed and cut into small pieces. Take 25g of the leaves into 100ml distilled water boiled for 2hrs at 90°C. The extract was filtered using what man filter paper. After the synthesis of nanoparticles, the filtrate was stored**.** To synthesis, the TiO_2 nanoparticles, dissolve 1.0 N of Titanium Chloride ($TiCl_4$) in 100 ml of Millipore water. Added leaves extract drop-wise under constant stirring up to achieve pH of the solution became 7. The mixture was subjected to stirring for 4 hours continuously. In this process, nanoparticles were formed, by using what man filter paper this nanoparticle was separated afterward and washed repeatedly with water to remove the by-products. At 100°C for overnight the nanoparticles were dried and calcined for 4 hours at 500°C (33).

Characterization techniques: the average crystalline size was measured by atomic force microscopy **(**AFM) illustrate 2D and 3D topological as show that the synthesized TiO_2 NPs are spherical and the size of an average diameter was 57.07 nm in figure (1: A). The morphology of the nanoparticles was observed by TEM-100 CXII Transmission electron microscope as shown in figure (1: B). The crystal structure was measured by X-ray diffraction pattern as shown in figure (1: C). By using Field emission scanning electron microscopy further characterization was done (FE-SEM) as shown in figure (1: D) (33, 34, 37).



2D



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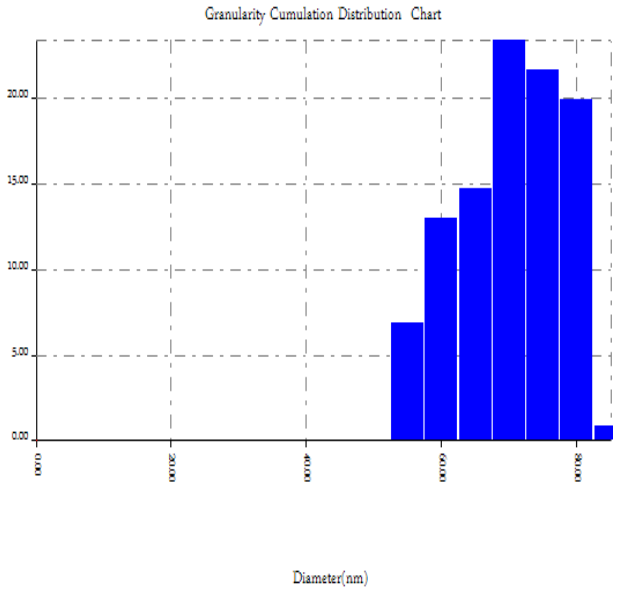
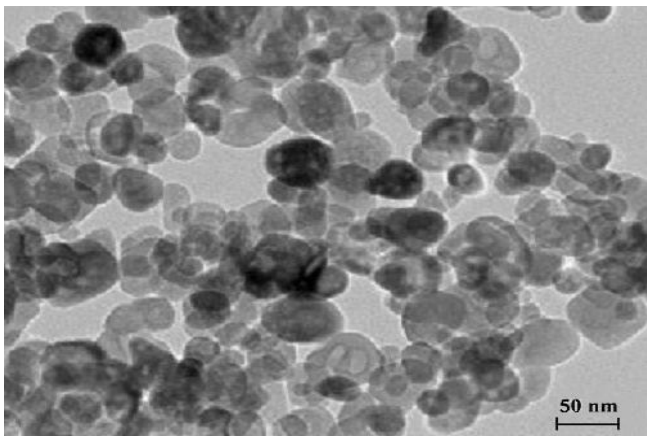


(A)



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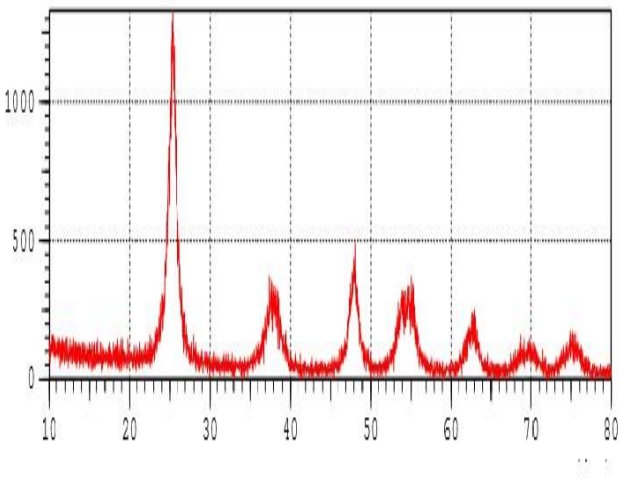
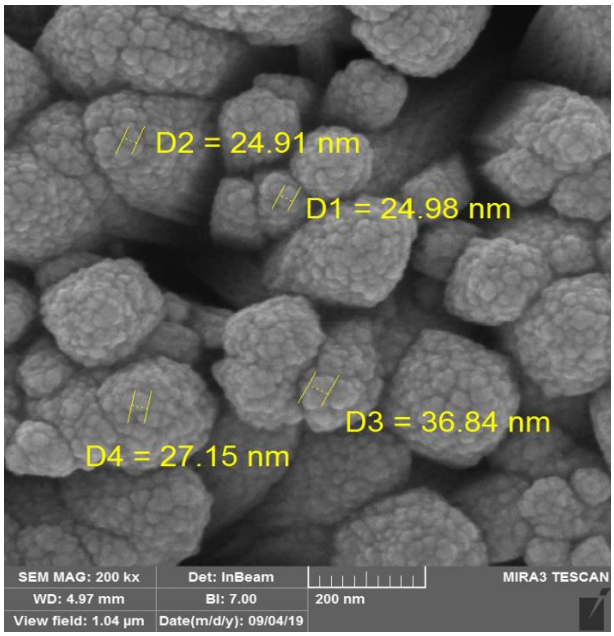


(C)



(D)

Figure 1. (A) Atomic Force Microscopy of TiO_2 NPs (ATM), (B): Transmission Electron Microscope (TEM), (C): X-ray diffraction (XRD), (D): Field Emission Scanning Electron Microscopy (FE-SEM).

Collection of bacterial isolates: bacterial isolates were provided from the medical laboratories of Al-Yarmouk Techeaing Hospital / Baghdad / Iraq. The isolates were characterized by using traditional culture media and biochemical tests. Final characterization was made by using API 20 E Kit. Bacterial inoculum prepared at a concentration of 1.5×10^8 CFU/ml and by using a hemocytometer the number of cells was calculated.

Used TiO_2 NPs in the production of prodigiosin and phenazine: The nutrient broth was used to achieve this procedure. Both the isolates were grown on poor non-selective nutrient broth. Different concentrations of TiO_2 NPs (0.005, 0.01, and 0.015) mg/l were added to the media, and the Erlenmeyer flasks were sealed with cotton-wool bungs and wrapped with aluminum foil. By autoclaving for 15 min at 121°C the flasks were sterilized. *Serratia marcescens* was grown at 50, 30, 40 °C for (24/48/72) hours of incubation to choose a better temperature for production, while *Pseudomonas aeruginosa* was grown at 37 °C for (24/48/72) hours according (36).

Extraction and quantification of prodigiosin and phenazine: 20 ml of acidified ethanol were added to 10 ml of the flask content at the end of the incubation period and put in a shaker at 3000 rpm for 15 min and to remove pigment-free pellet they centrifuged for 15 min. Prodigiosin quantification using relative prodigiosin concentration was expressed per cell by measuring the absorbance spectrophotometrically. As unit/cell the prodigiosin estimation was expressed after measuring the absorbance at 600 nm. While as unit/cell after measuring the absorbance at 690 nm at the end of incubation phenazine estimation was expressed.

RESULTS AND DISCUSSION

Morphology and cultural identification of bacterial isolates

The genus *Serratia* belongs to the Enterobacteriaceae family, which comprises 14 different species of bacteria. The characteristic feature of *S. marcescens* which differentiates it from other bacterial cells is the production of prodigiosin. The morphological diagnosis of these isolates is characterized by the appearance of the colonies in a bright red

color within the culture medium as in Figure (2) as well as the other distinctive characteristic of having a smell that resembles the smell of fish within the culture media. Serratia is characterized by its heat sensitivity to the growth and production of prodigiosin. The isolates were developed using more than one temperature (40, 30, 50) °C. The results showed that all isolates did not show any signs of growth at 50 °C while they grew relatively at 40 °C and without prodigiosin production. At 30 °C, isolates exhibited the highest rates of growth and production of prodigiosin (Figure 3). The *Pseudomonas* genus belongs to the Pseudomonadaceae family and has *P. aeruginosa* with the appearance of colonies in greenish-blue within the culture medium and are identified as usually and initially by the appearance of iridescent and the appearance of the aroma-like smell of grapes in the laboratory after incubating at 37 °C temperature for 24 hours (Figure 3). Then the most productive isolates of phenazine were chosen.

Increase the production of prodigiosin and phenazine using TiO_2 NPs: In this study, the isolates of the red dye prodigiosin and the green dye phenazine were selected, developed, and activated using the nourishing broth and after the addition of several concentrations of TiO_2 NPs (0.005, 0.01, 0.015) mg / L and at a suitable temperature for (24, 48, 72) hours. The results showed that the concentration (0.01) mg / L and after 72 hr. of incubation showed the highest yield of prodigiosin and phenazine compared to other concentrations as in Figures (4 and 5). The concentration of prodigiosin and phenazine was calculated using the colorimetric method. The cells were separated by precipitation, the leachate was taken and the absorbance was measured at 600 nm and 690 nm for prodigiosin and phenazine, respectively, as in Figures (6 and 7).

Figure 2. S. m*arcescen*s colonies on nutrient agar after incubation for 24 hours at 30 °C.

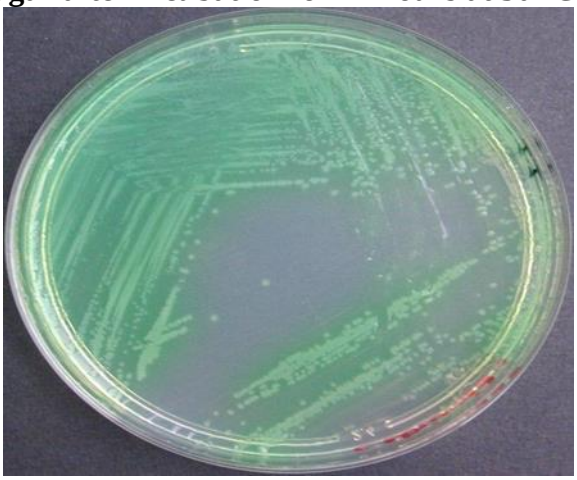


Figure 3. *P. aeruginosa* colonies on nutrient agar after incubation for 24 hour at 37 °C

Figure 4. S. m*arcescen*s isolates on poor non-selective nutrient broth after incubation with (0.01 mg/L) of TiO_2 NPs for 72 hours at 30 °C.

Figure 5. *P. aeruginosa* isolates on poor non-selective nutrient broth after incubation with (0.01 mg/L) of TiO_2 NPs for 72 hours at 30 °C.

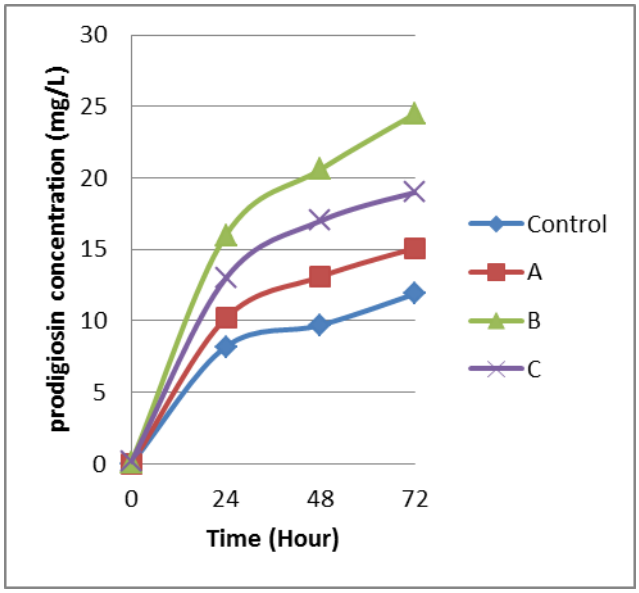


Figure 6. It represent the concentration of prodigiosin at different times (24, 48, 72) hours and with the using of different concentrations of TiO_2 NPs (A): 0.005 mg/L, (B): 0.01 mg/L, (C): 0.015 mg/L, in comparison with control (without TiO_2 NPs).

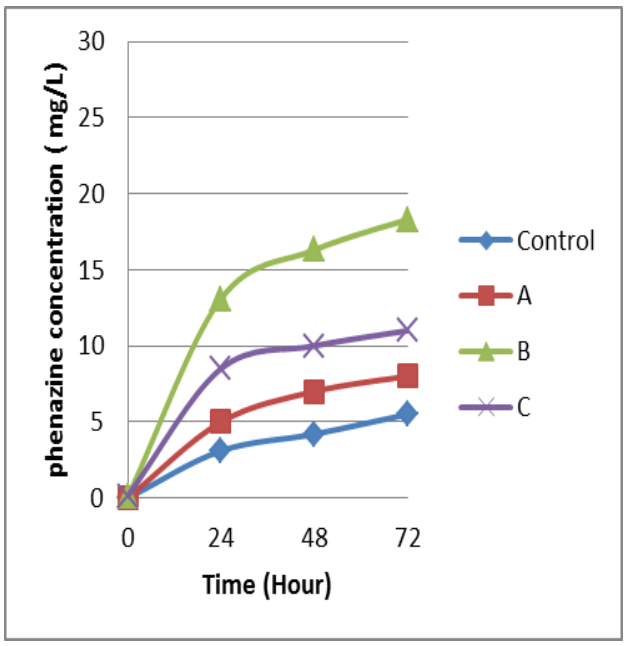


Figure 7. It represents the concentration of phenazine at different times (24, 48, 72) hours and with the using of different concentrations of TiO_2 NPs (A): 0.005 mg/L, (B): 0.01 mg/L, (C): 0.015 mg/L, in comparison with control (without TiO_2 NPs).

By lacking the complex immune system, many animals, plants and microorganisms, produce secondary metabolites including those that are harmful to a variety of other organisms. Although not essential in metabolic processes, these metabolic non-synthetic products appear to provide an action of defense against any attack and provide a competitive advantage upon the producer organism (1, 6, 9). Although each secondary metabolite has unknown ecology, laboratory assays have shown that such compounds possess antifungal (24), antibacterial (3, 7, 22, 37), and anti algal properties (17). Secondary metabolites have been traditionally mined from producing organisms for use in the drug production industry due to their biological properties, Providing an assortment of diverse biochemical structures selected over geologic time, natural or non-synthetic products are the most confidently successful source of drug leads (8, 25), which showed pathogens killing by different mechanisms. Pharmacologically penicillin and vancomycin as antibacterial secondary metabolites inactivate bacterial cell wall synthesis (10, 15) while tetracycline and erythromycin inactivate bacterial protein synthesis (12). By binding steroidal alcohols in

the fungal membrane, amphotericin B showing a potent fungicide activity (24). Although, the search for bactericidal and/or fungicidal natural products receiving competition from combinatorial chemistry approaches continues to show a viable source of medication needs (9). According to the above, we had design our experiment to increase the production of prodigiosin and phenazine. As mentioned previously, they are considering a promising compound since they showed antibacterial, antifungal, antitumor, and immunosuppression activities. Using TiO_2 NPS is considered a promising novel approach to enhance the production ability of bacterial isolates. Although different concentrations were used in this study, results indicate that (0.01 mg/L) was the best one to increase the production of both prodigiosin and phenazine. Lower concentration (0.005 mg/L) showed a slight increase in production. Moreover, (0.015 mg/L) concentration showed a lower effect in production activity in compression with (0.01 mg/L) which may suggest it caused toxicity to bacterial cells and kill them. In addition to that this research give also a promising idea of using non-selective cheap media for production to reduce the cost of production of such important compounds.

CONCLUSION

Based on the study, it was concluded that the (0.01 mg/L) of TiO_2 nanoparticles was found to be the best for prodigiosin production by *S. marcescens* as well as phenazine production by *P. aeruginosa*. Therefore, the TiO_2 nanoparticles can be suggested to enhance the production activity for `secondary metabolites by other microorganisms. Also using the poor non-selective nutrient broth for production was shown to be very important for economic issues.

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