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Biosynthesis of Nio Nanoparticles Using Prodigiosin Pigment and its Evaluate of Antibacterial Activity Against Biofilm Producing MDR-*Pseudomonas Aeruginosa*

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ABSTRACT

The entire investigation's focus was on the production of nickel oxide nanoparticles (NiONPs), using prodigiosin pigments produced by *Serratia marcescens* as a stabilizing and reducing agent. Nickel oxide nanoparticles are synthesized using nickel sulfate $NiSO_4$ (10mg) with a concentration of prodigiosin (10g/100ml). Biosynthesized NiO nanoparticles have been characterized by using many techniques, such as (UV-Vis, AFM, XRD, FTIR, and FE-SEM). The AFM analysis revealed that NiONPs have an average diameter size of (41.77 mm), and the FE-SEM Image displays Spherical. Additionally, the effect of NiONPs with different concentrations on the bacteria *Pseudomonas aeruginosa* was measured and the inhibition zone reached to (29 mm). Biosynthesis of NiONPs using prodigiosin was shown to have promising activity as an antibacterial against the biofilm-producing *Pseudomonas aeruginosa*.

Keywords: Biofilm, Antimicrobial activity, Prodigiosin, Nickel oxide nanoparticles (NiONPs).

التخليق الحيوي لدقائق اوكسيد النيكل النانويه باستخدام صبغة البروديجيوسين وتقييم الفعاليه الضد بكتيريه *Pseudomonas aeruginosa* المنتجه للغشاء الحيوي الرقيق

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الخلاصه

هدفت الدراسة الحاليه للتركيزعلى طريقة التخليق الحيوي لجسيمات أكسيد النيكل النانوية (NiONPs) باستخدام صبغة بروديجيوسين المنتجه من *Serratia marcescens* كعامل استقرار واختزال. تم تصنيع الجسيمات النانوية من أكسيد النيكل باستخدام كبريتات النيكل $NiSO_4$ (10غم) مع تركيز من البروديجيوسين (10غم / 100مل). وتم توصيف جسيمات NiO النانوية بعدة طرق مختلفه (UV–Vis و AFM و XRD و FTIR و FE–SEM). كشف تحليل AFM أن NiONPs يبلغ متوسط حجم قطرها 41.77 مليمتر، وتعرض الصورة FE–SEM صورة كروية. بالإضافة إلى ذلك ، تم قياس تأثير NiONPs بتركيزات مختلفة

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على بكتيريا *Pseudomonas aeruginosa* ووصلت منطقة التثبيط إلى 29 مم. أظهر التخليق الحيوي لـ NiONPs باستخدام البروديجيوسين نشاطًا واعدًا كمضاد للبكتيريا ضد إنتاج الغشاء الحيوي *Pseudomonas aeruginosa*.

INTRODUCTION

Nanotechnology is the study of extremely small structures mostly with sizes ranging from (1 to 100 nm), which also provides them with exceptional properties when compared to the same bulk-size particles [1, 2]. Nikel Oxide nanoparticles (NiONPs) have gained popularity due to their wide range of characteristics such as transfer of electron capability, super-capacitance, electro-catalysis, and excellent chemical stability [3]. Mostly, as a result, numerous physical and chemical approaches have also been effectively used to produce NiO NPs [4, 5]. High surface area NiO nanoparticle films have also been created using processes such as laser liquid ablation, electrodeposition, spin coating process, chemical bath deposition, as well as spray pyrolysis [6, 7].

Biosynthesis of NiO by bacteria and fungi extract supplies more advantages than chemical methods and physical methods since it is simple to process, very cost-effective, and scalable for large-scale production [8, 9]. This process did not require high pressure, expensive machines, high temperatures, and toxic chemicals. Prodigiosin, a tripyrrole red linear pigment, is secreted normally as a secondary metabolite of microbes. The pathogenic bacterium *Serratia marcescens* is the native producer [10]. Numerous *Serratia marcescens* isolate as well as other Gram-negative bacteria including *Vibrio psychroerythrus* and *Hahella chejuensi* generate prodigiosin [11, 12]. A collection of bacteria known as a biofilm often lives on surfaces and is covered in an extracellular matrix [13, 14]. In comparison to their planktonic cousins, biofilms exhibit many distinct differences. *Pseudomonas aeruginosa* is a bacterium that is known to create powerful biofilms, which are pervasive in the environment and have a significant impact on our lives in both positive and negative ways [15, 16]. *Pseudomonas aeruginosa* biofilms in immune compromised patients, especially those with wound infection and cystic fibrosis, led to serious issues. Additionally, the distinct characteristics of biofilm make it more difficult to get rid of the infection, which promotes the development of chronic infections [17, 18, 19].

Materials and methods

Preparation of production media: In a typical procedure, Fermentation media preparation is based on [20]. Medium prepared by mixing components such as Peptone (5 g/L) as nitrogen, sucrose (10 g/L) as carbon source, $MgSO_4.7H_2O$ (0.61 g/L), $MnSO_4.4H_2O_2$ (2 g/L), $CaCl_2.2H_2O$ (8.82 g/L) and $FeSO_4.4H_2O$ (0.33 g/L). After setting the pH at 7.0, the medium was sterilized by autoclaving at 121° C for 15 minutes. After the medium had been sterilized and cooled, it was injected with 2% of the selected bacterial isolate (0.5 McFarland standard equals $1.5x10^8$ CFU/ml), which was then grown for 72 hours at 120 rpm in a shaker incubator at 28 °C. [21]. All Materials were obtained from Sigma, Ltd., USA)

Prodigiosin pigment production and purification: After incubation, 250 ml of cell-free broth culture from *S. marcescens* was recovered, from which raw prodigiosin was extracted. For 30 minutes, the culture medium was centrifuged at 8000 rpm. The extracted cell was mixed well with 250 ml of methanol for three hours at room temperature before the supernatant was discarded. The resulting mixture was then centrifuged for 20 min at 8000 rpm, collecting and filtering the supernatant through a filter paper (0.2 μm, Whatman). A rotary evaporator was used to concentrate the methanol filtrate at 70˚C and twice the amount

of chloroform was then added to extract the red pigment. The two solvents were mixed vigorously in a separator funnel. The chloroform phase (organic phase) was collected and dried by using a rotary evaporator. Then, the powder produced by the evaporation of chloroform was collected and stored in dark containers representing the prodigiosin pigment [10].

Biosynthesis of nickel oxide nanoparticles:

All of the ingredients and reagents required for the creation of NiO nanoparticles were purchased from Sigma, Ltd., USA, and the manufacture of NiO NPs was carried out following the instructions provided by [8] just slightly modified The ultrasonication bath was used ~~to~~ disperse 10mg of prodigiosin pigment for 30 minutes after it had been dissolved in 100ml of deionized distilled water. Following the addition of 10g of nickel sulfate NiSO4, which was dispersed for 30 minutes in an ultrasonic bath, the mixture was placed in a shaker incubator where it was left for the night at room temperature.

After the creation of nanoparticles, they were repeatedly rinsed with deionized distilled water to remove any leftover by-products before being separated from prodigiosin pigment and other components using a centrifuge. Then, various concentrations of the synthesized NiO NPs (50, 100, 150, 200, and 250 μg/ml) were made.

Characterization NPs Techniques:

The average crystalline size of the generated NiO NPs was determined to display the 2D and 3D topologies. using atomic force microscopy (AFM), and the crystal structure was determined using X-ray diffraction patterns. Using field emission scanning electron microscopy, more characterization was accomplished (FE-SEM). NiO NPs were created and characterized in the Biotechnology Department, Nanotechnology Laboratory, College of Science, University of Baghdad, Iraq, and the Chemistry Department, College of Science, University of Baghdad, Iraq [3].

Antibacterial test (*in vitro*):

Through the agar, well diffusion technique, the minimal inhibition concentration (MIC) of NiO NPs was carried out, and the antibacterial activities of the biologically synthesized NiO NPs were tested against *Pseudomonas aeruginosa* (local isolate (as pathogenic isolate taken from human) provided from the biotechnology Department, College of Science, and University of Baghdad, Iraq). Biochemical tests and morphological characteristics were performed and identified using automated methods (Vitek II system) shown in (Figure 1) [22]. The Petri dishes were also given a 25 ml application of sterile Muller Hinton agar medium, which was then let to set in a lab environment. The developed test species were cultured on the agar medium using sterile cotton swabs. To the already-formed wells, various NiO NPs concentrations (50, 100, 150, 200, and 250 μg/ml) were administered. The plates were then infected and kept at a temperature of 37 °C for 24 hours. The area around the tested wells where there are inhibitions was measured.

bioMérieux Customer: **Microbiology Chart Report** Printed Sep 1, 2021 21:33 CDT

Patient Name: Patient ID:
Location: Physician:
Lab ID: 03 Isolate Number: 2

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:**	4.82 hours	**Status:**	Final
Selected Organism	99% Probability **Bionumber:**	**Pseudomonas aeruginosa** 0003053003500000		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	+	21	BXYL	-	22	BAlap	+
23	ProA	+	26	LIP	+	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	-	57	BGUR	-
58	O129R	-	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Figure 1: Vitek II for *Pseudomonas aeruginosa.*

Results and Discussion

Due to the widespread use of green nanoscience and technology in domains including response stimulation, medicine transport, and sensor manufacture, these sectors have recently undergone rapid growth [19, 23]. Studies were focused on finding substitute medications with specific antibacterial and anticancer properties that were more readily available, less expensive, and had fewer adverse effects. Today's nanoscale metrology faces a significant barrier in the nanomolecular analysis [2].

Production of prodigiosin pigment:

The formation of prodigiosin began after a 12-hour incubation period. At 48 hours of incubation, the prodigiosin concentration (at the end of the exponential phase) was determined to be 0.35 g/L, and at 72 hours (during the stationary phase), it was 0.49 g/L. The medium's transition to red color signaled the formation of prodigiosin, which was predominantly collected during the stationary period [24].

Characterization of prodigiosin pigment

UV-visible spectrophotometer:

The absorbance properties of the prodigiosin that was extracted by *S. marcescens* were measured using a UV-Vis spectrophotometer (Shimadzu, Japan) with a range of 400-600 nm (Figure 2). In particular, and in good accord with other research, the highest absorption was seen at about 539 nm [25].

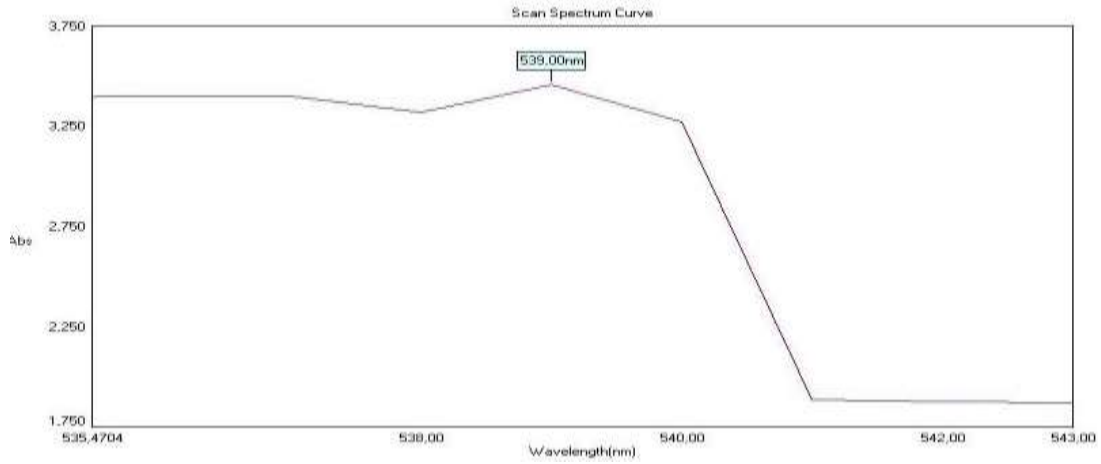
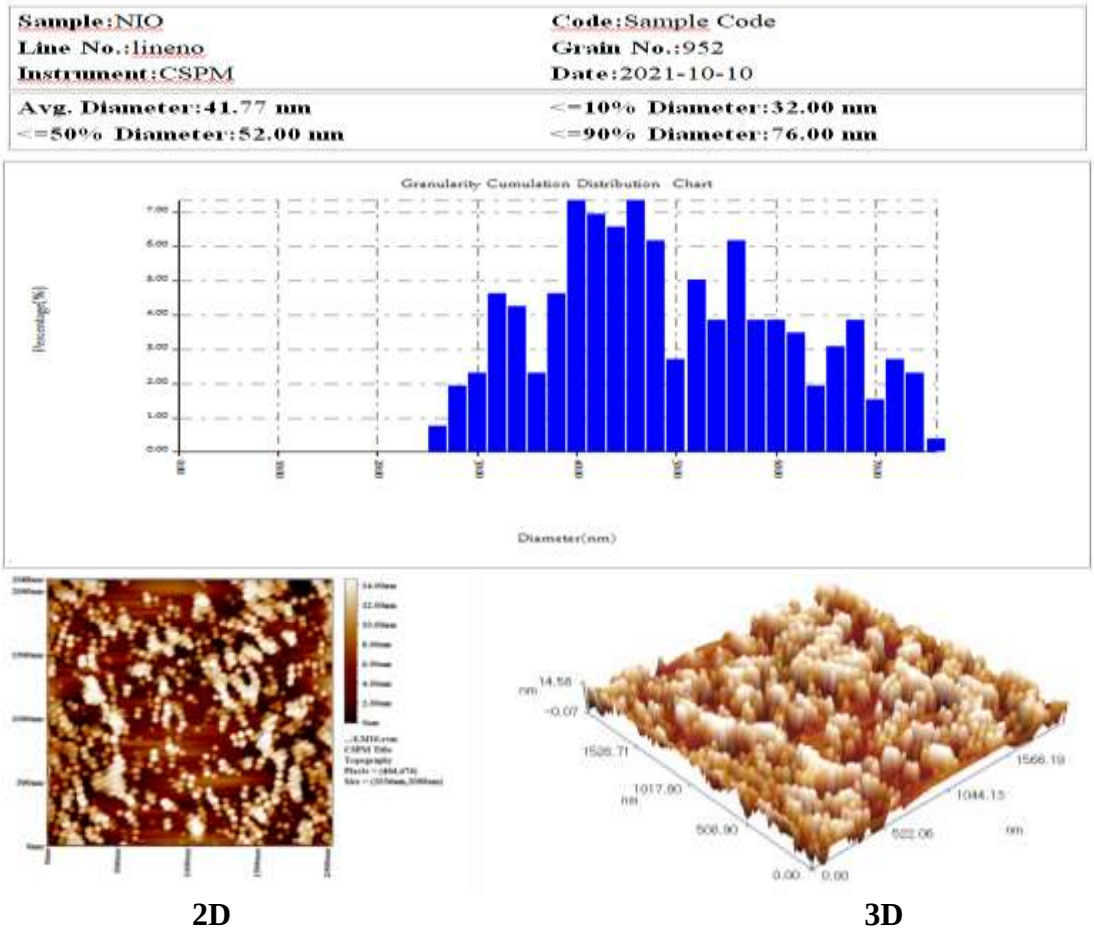


Figure 2: Absorption spectra of the purified prodigiosin pigment.

Atomic force microscopy (AFM):

Atomic force microscopy (AFM) (Avg. Diameter: 41.77 nm) can be used to quickly assess the nanoparticles' sizes by taking their height into account. The image from the AFM displays two- and three-dimensional (3D) data for NiO NPs, allowing for the quantitative measurement of the nanoparticles' height. The findings of the present study's AFM analysis were shown in (Figure 3).



2D **3D**

Figure 3: Average size of NiO NPs Synthesized using Prodigiosin illustrate 2D and 3D topological by (AFM)

X-ray diffractometer:

The XRD pattern of NiO nanoparticles was obtained from biosynthesis as shown in (Figure 4). XRD patterns show that all NiO NPs these findings The peaks positions appearing at 2θ = 20.23◦, 21.76◦, 24.34◦, 30.48◦, and 39.54◦ can be readily indexed as (111), (200), (220), (311), and (222) crystal planes of the bulk NiO, respectively [3].

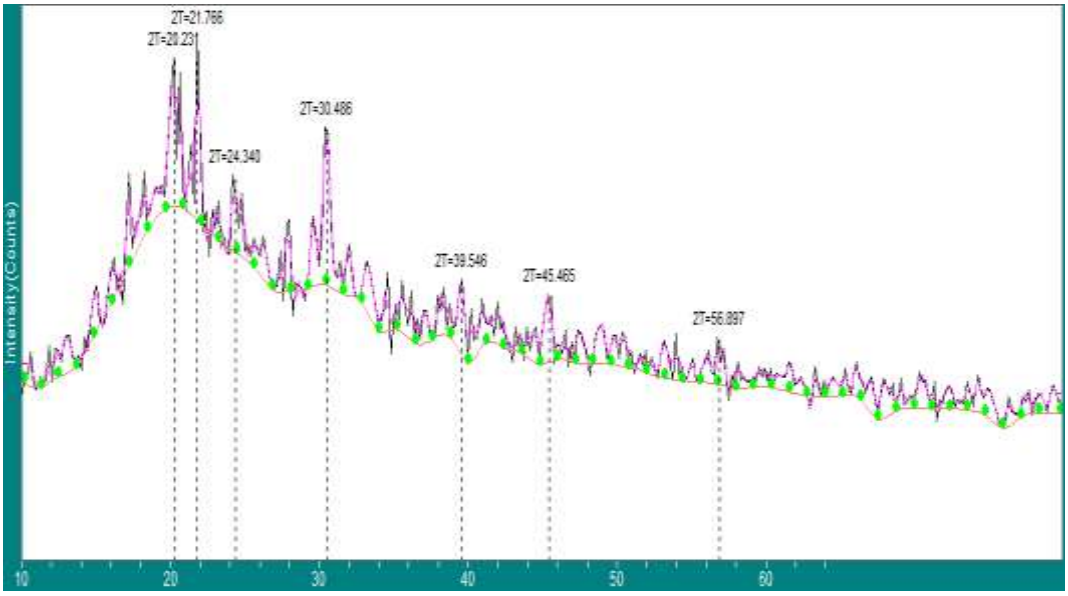


Figure 4: XRD images of NiO nanoparticles**.**

Field Emission Scanning Electron Microscope (FE-SEM):

NiO nanoparticles' FE-SEM pictures revealed signs that the particles' shape is spherical. The produced NiO NPs' morphology was depicted in (Figure 5) based on the results of the FE-SEM analysis, which was in strong agreement with the AFM findings.

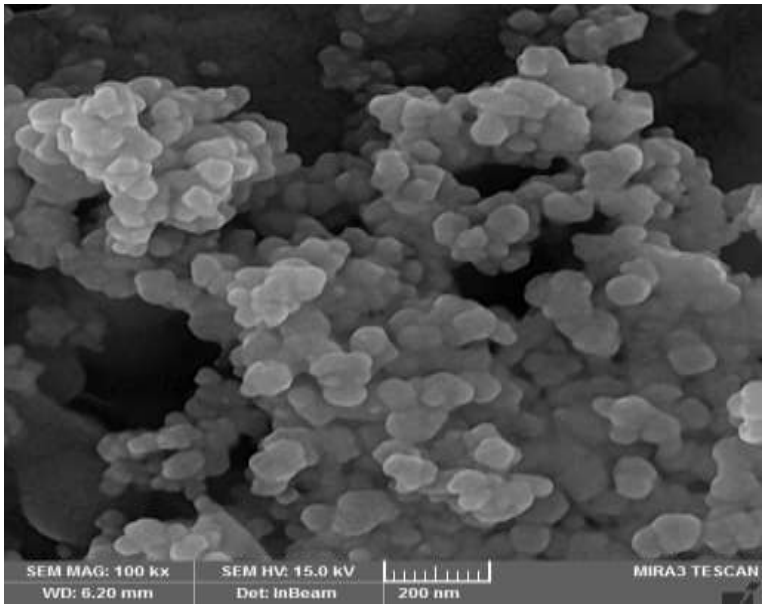


Figure 5: FE-SEM Images of NiONPs Synthesized using Prodigiosin.

Antibacterial susceptibility test:

It is demonstrated ~~in~~ that bio-synthesised NiO nanoparticles have antibacterial activity at values of 50, 100, 150, 200, and 250 µg/ml (Table 1). The antibacterial activity of NiONPs is

clearly demonstrated to be directly dependent on the NiO concentrations used, even at concentrations as low as 50 g/ml in an inhibition zone of 10 mm, while a NiO concentration of 250 µg/ml produced an inhibitory zone of 29 mm. The size of the inhibitory zone may depend on the bacteria's susceptibility as well as the several ways that NiO interacts with the chosen bacterium. Reactive oxygen species (ROS) production is the primary cause of NiONPs' harmful effects on bacteria in general. Damage to cellular components such as DNA, proteins, and lipids is specifically blamed for ~~the~~ ROS toxicity to the cell wall. ROS production is generally regarded as the key component of antibacterial activity linked to NiO phototoxicity [26]. This then causes oxidation, which inhibits or eliminates the microbes. The MIC was assessed using the serial dilution method with a range of 50-250 g/mL in accordance with the CLSI [27, 28]. Therefore, the antibacterial activity of NiONPs is crucial due to the harmful bacteria's capacity to enter the environment's food chain [9].

Table 1: Inhibition zones of NiO nanoparticles against Mdr- *Pseudomonas Aeruginos.*

NiO NPs concentration (µg/mL)	Inhibition zone (mm)
50	10
100	14
150	19
200	22
250	29

CONCLUSION

This study effectively demonstrated the production of NiO nanoparticles using purified prodigiosin pigment as a reducing agent. Green synthesis of nanoparticles uses reagents that are safe, non-toxic, and kind to the environment [29]. At 72 hours, the reducing agent's (prodigiosin) appropriate concentration was (0.49 g/L). AFM, XRD, FT-IR, and FE-SEM techniques were also used to characterize the studied NiONPs. In particular, the synthesized NiONPs displayed spherical particles, which were proven by the FE-SEM, while the XRD patterns showed successful NiONPs phase development. While the AFM showed a 41.77 nm average diameter. The bio-synthesised nanoparticle was found to have potent antibacterial activity against the tested microorganisms in the antibacterial activity test. The maximal inhibitory zone at a concentration of 250µg/mL was discovered to be 29 mm. The difference in inhibition diameter may be due to different interactions between NiONPs and the microorganism, and due to the susceptibility of bacteria used in the current study [28, 30]. The main mechanism of NiONPs toxicity is potentially associated with metal oxides carrying the positive charge even though the microorganisms bear negative charges; this results in electromagnetic interaction between microorganisms and metal oxides leading to oxidation and finally death of microorganisms [4, 27]. The bactericidal action of NiONPs on bacteria is of extreme importance due to the ability of pathogenic bacteria to join the food chain of the ecosystem [31]. The antibacterial activity of NiONPs was due to the capability of NiONPs to cause free hydroxyl radicals (OH^-) [5]. The antimicrobial effect of NiONPs against fungi and bacteria has been demonstrated and communicated in last year's research.

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