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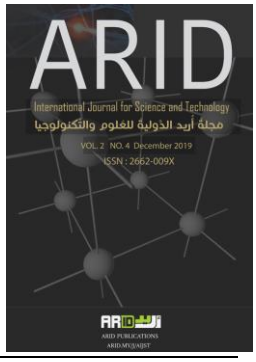

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Synthesis, Characterization, Optimization of Ag-Nanoparticles and Recognition of Nitrate Reductase Enzyme in Clinical Isolates of *Escherichia coli*

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تخليق ، توصيف ، وتحسين الجسيمات النانوية للفضة والتعرف على الانزيم المختزل للنتريت في العزلات السريرية التابعة للـ *Escherichia coli*

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Abstract

Biological methods are favorite for synthesis of nanoparticles because of environmental sustainability and cost efficiency. Different types of bacteria have a large part in the synthesis of metallic nanoparticles, and nitrate reductase enzyme, which catalyzes the reduction of nitrate to nitrite, can be produced by various species of prokaryotes like *Escherichia coli*. Results of Chrome agar medium showed that *Escherichia coli* as green-blue colonies, and the confirmation of bacterial identification was done by using VITEK-2 compact system. Outcomes indicate that 37ºC, pH 9.0 and 10 milli- Molar of solid silver nitrate considered the optimal conditions for synthesis of silver nanoparticles. Color of *Escherichia coli* culture supernatant when mixed with silver nitrate and glucose was changed from light yellow to dark brown after 24h incubation as a pre-confirmation of synthesis of silver nanoparticles. Particle size analysis exhibited that silver nanoparticles size ranged from (10.3 to 86.0) nanometer in diameter, and the mean size is 40.2 nanometers, while Scanning electron microscope showed less aggregation of silver nanoparticles with spherical particles. Fourier Transform Infrared analysis of silver nanoparticles showed that strong bands were at 3398.57, 2966.52, 1348.24, 1045.42, 671.23 cm^{-1} and 532.35 cm^{-1} corresponds to OH-free, H-C-H, OH-bend resembling to phenol compounds, C-O, C-H and silver nanoparticles bends. Amplification of nitrate reductase genes showed that *napA1* and *napA2* genes were found in 100% and 98 % of testing isolates, respectively. From these results, it was obvious that bacterial enzyme can apply in the reduction of metals and product nanomaterials in different environments, and this biosynthesis had many advantages in biological and pharmaceutical applications.

Key words: *Escherichia coli* bacteria, silver nanoparticles synthesis, nitrate reductase genes.

الملخص

تُفضل الأساليب البيولوجية في تصنيع الجزيئات النانوية بسبب الاستدامة البيئية وفعالية التكلفة. الاحياء المجهرية ، ومنها البكتيريا لها دور كبير في تخليق الجسيمات النانوية المعدنية ، ويمكن أن تنتج انزيم مختزل النتريت ، الذي يحفز اختزال النترات إلى النتريت ، بواسطة أنواع مختلفة من بدائية النواة مثل الـ *Escherichia coli*. اظهر وسط الكروموا كار البكتريا كمستعمرات خضراء مزرقه ، ثم أكد التشخيص باستخدام نظام VITEK-2. تشير النتائج إلى أن 37 درجة مئوية ، درجة الحموضة 9.0 و 10 مل مولار من نترات الفضة الصلبة اعتبرت هي الظروف المثلى لتصنيع الجسيمات النانوية الفضية. تم تغير لون رائق بكتريا القولون مع نترات الفضة والجلوكوز من الأصفر الفاتح إلى البني الداكن بعد 24 ساعة من الحضانة كتأكيد لانتاج الدقائق النانوية للفضه. أظهر تحليل حجم الجسيمات النانوية للفضة يتراوح بين (10.3 إلى 86.0) نانومتر في القطر ، والحجم المتوسط هو 40.2 نانومتر ، في حين أن المجهر الالكتروني الماسح أظهرها كجزيئات كروية ذات تجمع قليل. أظهر تحليل Fourier Transform Infrared للجسيمات النانوية أن اعلى حزم كانت عند 3398.57 و 2966.52 و 1348.24 و 1045.42 و 671.23 cm-1 و 532.35 cm-1 والتي تتوافق مع -OH و HCH و OH-bend التي تشبه مركبات الفينول ، CO، CH تخص جزيئات النانو للفضة. أظهر تضخيم جينات اختزال النترات أن جين napA1 موجود بنسبة 100٪ و napA2 في 98٪ من عزلات الاختبار. يمكن أن يستخدم هذا الإنزيم في اختزال المعادن في البيئات المختلفة وتشكيل الجسيمات النانوية واستخدامها للتطبيقات البيولوجية والصيدلانية.

الكلمات المفتاحية: بكتريا *Escheichia coli* ، تصنيع الدقائق النانوية للفضة، جينات انزيم اختزال النتريت.

1. Introduction

E.coli is a gram–negative, rod shaped bacterium, facultative anaerobic, [1]. Usually E.coli forms a beneficial symbiotic relationship through, its host and plays important roles in promoting, the stability of the luminal flora and in maintaining normal intestinal homeostasis [2]. Virulent strains of E.coli can cause a variety of diseases in human gastroenteritis, urinary tract infection and neonatal meningitis .[3]

The microorganisms play a significant role in the handling of toxic metals through reduction of metal ions and employment as interesting nanofactories [4]. Bacteria being prokaryotes have survived the test of time in enriching ions synthesizing magnetite nanoparticles, reducing Ag into metal particles, Biosynthesis of silver nanoparticles by E. coli occurred in the presence of Nitrate reductase enzyme (NR) .[5]

Nitrate reductase an enzyme that converts nitrate to nitrite, [6]. According to the definitions, enzymes are protein molecules which can produce by different Prokaryotes and Eukaryotes (including bacteria, fungi and blue-green algae) [7]. Forming nanoparticles , Nitrate reductase is located in the periplasm (Nap) [8]. The physiological role of Nap is acting as a mediated enzyme in anaerobic respiration, a periplasmic nitrate reductase (Nap) contains four basic components in many bacteria: napB, napA, napD nd napC, while the E.coli contains three additional genes: napF, napG and napH [6]. The aim of this study was to detect the presence of periplasmic nitrate reductase enzyme in E.coli during biosynthesis of silver nanoparticles from supernatants of this bacteria.

2. Experimental procedure

2.1 E. coli Isolates

Bacterial isolates obtained from the Microbiology laboratory in the Department of Biology at Mustansiriyah University (Sixteen isolates from different clinical sources (wounds, urine, stool, blood

and burns). Chroma agar medium and Vitek-2 system were used to confirm the characterization of bacterial isolates.

2.2 Ag-Nps synthesis by using culture supernatant of E.coli

Bacterial supernatant mixed with 10 mM of silver nitrate ($AgNO_3$) and 100 mM of glucose in 1:1:1 portion, according to method modified by [9].

2.3 Ag-Nanoparticles Characterization

2.3.1 Scanning Electron Microscope (SEM)

SEM (TESCAN-VEGA/USA) with resolution 3nm at 30 KV used to determine the aggregation and the shape of Ag-Nps.

2.3.2 Particle size Distribution

Particle Sizing Software (Version 5.34) used to determine Ag-nanoparticles size.

2.3.3 Fourier Transform Infrared (FTIR) analysis

Silver nanoparticles (Ag-Nps) approved by FTIR -8400S (SHIMADZU) apparatus at a range of 4000–500 cm^{-1}.

2.4 Optimization of the Ag-Nps synthesis conditions

The effect of three variable parameters ($AgNO_3$ concentration, pH, and temperature), on the production of Ag-NPs optimized by varying one parameter at a time, such as the substrate concentration (1, 5 and 10) mM of solid $AgNO_3$, pH (3, 5, 7, 9, and 11), and temperature, (27, 37, 47 and 57°C). Biosynthesis of Ag-NP by *E. coli* occurs within the best condition of temperature, PH and concentration of $AgNO_3$ [10].

2.5 *Nitrate reductase genes amplification*

2.5.1 Primer Design

The primer sequence of nitrate reductase genes of *E.coli* was designed according to software of National Centre for Biotechnology Information (NCBI), and the accession no. of *napA1*was (H6E2348/69) (M.W.201pb), while *napA*2 was (H10407) (235pb) as in Table (1). They analyzed by primer-3 web

(version 40.0) software, then the primers prepared according to the manufacturing companies instructions (Alpha DNA).

Table (1): The oligonucleotide primer sets *napA1* and *napA2* genes

Gene name	Primer sequence	Product size
napA1	F-ACGGTAAAGACCGTTTGACG	201bp
	R- CGGCATAACCTTCCCAGATA	
napA2	F-TGGAAAAAGCAGCGAAGAAT	235bp
	R-ACCAGGTTGTTAGCCCACAC	

2.5.2 DNA Extraction:

Bacterial DNA extraction was carried by using (Genomic DNA purification Kit (Wizard® Promega, USA). Isolates inoculated on nutrient agar for 24hr at 37 ˚C, then harvested and suspended in 1 ml of LB broth in Eppendrof tube and mixed gently. The extraction of genomic DNA was performed according to the manufacturing of the company [11].

2.5.3 Polymerase chain reaction (PCR)

The primers diluted by adding nuclease free water according to the manufacture companies' information (Advanced Scientific Bureau). The amplification performed in a thermal cycler (BioRad (T-100). For amplification of *nap*A gene, five micro liters of the DNA were mixed with a PCR mixture that composed from 12.5 µl GoTaq Green Master Mix (2x), 1.5 µl from each primer of *napA1* gene and primer of *napA2* gene (10 µM concentration for each), then 4.5µl of nuclease free water to get final volume 25 µl.

2.5.4 Amplification reaction conditions

In the present study, 30 cycles of amplification used for *nap*A1 and *nap*A2 Table [2]. The cycling conditions applied according to [12] with modification.

Table (2): PCR Thermo cycling condition *napA1* and *napA2*

Condition	emperature	Time	No. of cycles
nitial Condition	94 º C	4 min	1
Denaturation	94 º C	1 min	30
Annealing	60 º C	30 Sec	
Extension	72 º C	30 Sec	
Final Extension	72 º C	10 min	1

3. Results and Discussion

E.coli colonies appeared as green-blue on CHROM agar medium, Figure (1). And this result due to the hydrolysis of chromogenic substrate including in the medium, which confirms appearance of *E.coli* colonies according to manufacturer's instructions. After that, positive isolates belong to *E.coli* confirmed by using the VITEK-2 compact system.

Figure (1): *E.coli* on chrom agar, A- without supplement. B- with supplement

According to [13] showed that CHROM agar STEC improved significantly screening for STEC compared to the conventional culture method with sorbitol-MacConkey agar, providing assistance, based on its fluorescence property, with detection of STEC co-infections and enabling strain isolation for further investigations, when required.

3.1 Optimizations of the Ag-NP synthesis conditions

The silver ion reduction was examined at different temperature degrees (27, 37, 47 and 57) ºC, and the optimum temperature for reduction was 37ºC; while the best substrate concentration was 10 mM of solid $AgNO_3$, and the pH 9.0 was the best for synthesis of silver nanoparticles.

Results of reference [10] found that the maximum absorbance of the reaction mixture had increased from 20 to 70 ℃, whereas it had decreased from 70 to 90 ℃.

Reference [14] observe that the maximum absorbance had increased when pH increased from (6-12). The results indicate that an alkaline pH favored for the formation of Ag-NPs, and showed that reduction result of metallic ions is sensitive to the pH of a solution [15].

3.2 Biosynthesis of Ag-Nps

Color of reaction mixture *(E.coli* culture supernatant with silver nitrate and 10mM glucose) was changed from light yellow to dark brown after 24h incubation. This result considered as pre-confirmation of Ag-Nps synthesis comparing to control as shown in Figure (2).

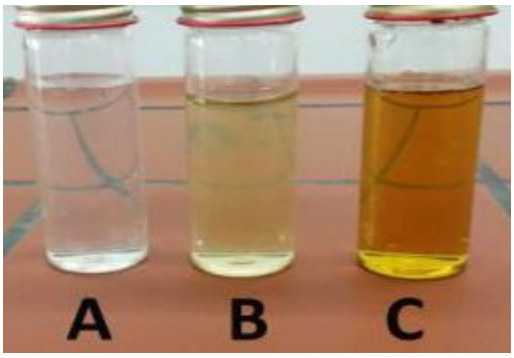

Figure (2): Synthesis of Ag-Nps (a) control (silver nitrate), (b) culture supernatant of *E.coli* (c) mixture of silver nitrate with bacterial supernatant and glucose

The strong color change suggests that the synthesis of silver nanoparticles could be better in adding of glucose [16]. Also references [17 and 18] showed that glucose used as reducing agent because of the encapsulation effect of glucose and trapping the Ag-NPs inside in glucose**.**

3.3 Characterization of synthesized silver nanoparticles

3.3.1 SEM analysis

Silver- Nps morphological features were studied by SEM, and result shown in Figure (3). SEM analysis showed that less aggregation of Ag-NPs with spherical particles, and the mean size is 40.2 nm.

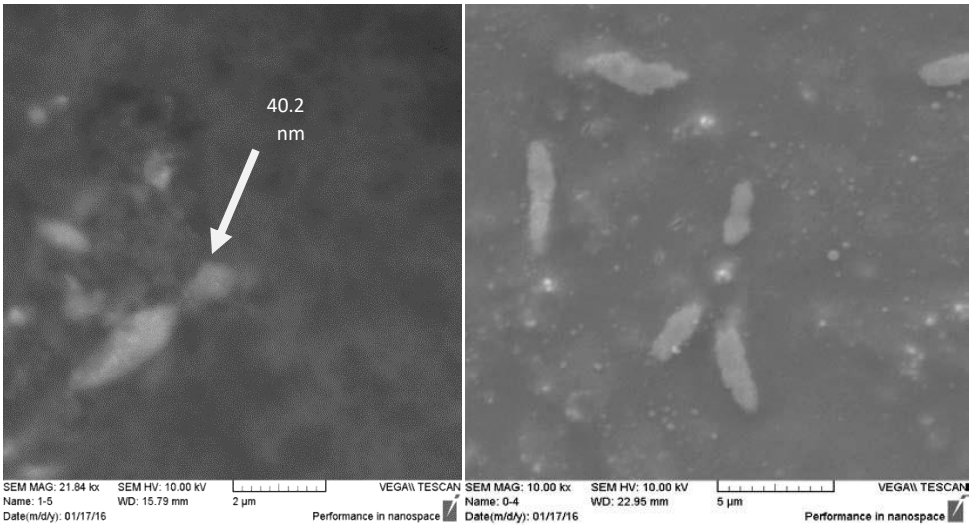

Figure (3): SEM pictures of biosynthesized Ag-NPs

The SEM was used to analyze shapes of nanoparticles, which synthesis by bacteria and showed that the synthesized Ag-NPs are spherical in shape for *E.coli* and other types of bacteria like *Pseudomonas aeruginosa* [19], *Bacillus thuringiensis* [20], *and Deinococcus radiodurans* [21].

3.3.2 Particle size analysis

Silver nanoparticles size ranged from (10.3 to 86.0) nm in diameter Figure (4). Size of Ag-NPs synthesized could be attributed to different cell growth and metal incubation conditions [20].

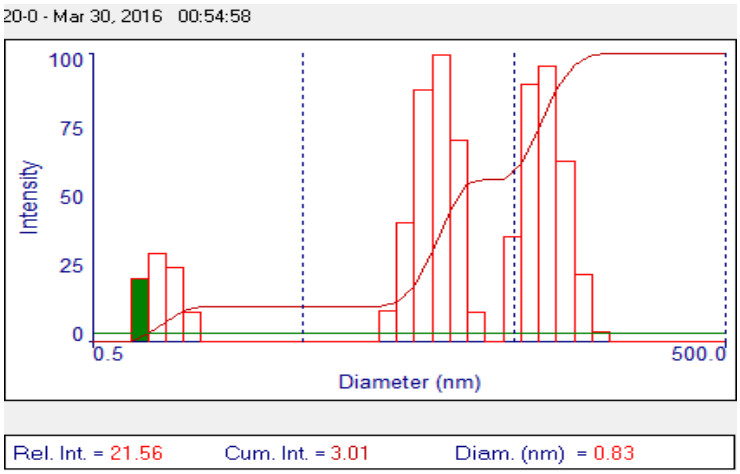

Figure (4): Particle size analysis of biosynthesized Ag-Nps

3.3.3 FT-IR analysis

The result of the FTIR spectrum for analysis Ag- nanoparticles synthesized by *E.coli* was shown in Figure (5). It showed that strong bands at 3398.57 cm^{-1} which corresponds to OH-free, 2966.52 cm^{-1} corresponds to H-C-H, while band at 1348.24 cm^{-1} corresponds to OH-bend resembling to phenol compounds can possibly persuade the synthesis and stability of Ag-NPs. The band of 1045.42 cm^{-1} corresponds to C-O Stretch, and the band at 671.23 cm^{-1} corresponds to C-H stretching of phenyl ring of substitution band, whereas the stretch for Ag-NPs established around 532.35 cm-^{1}.

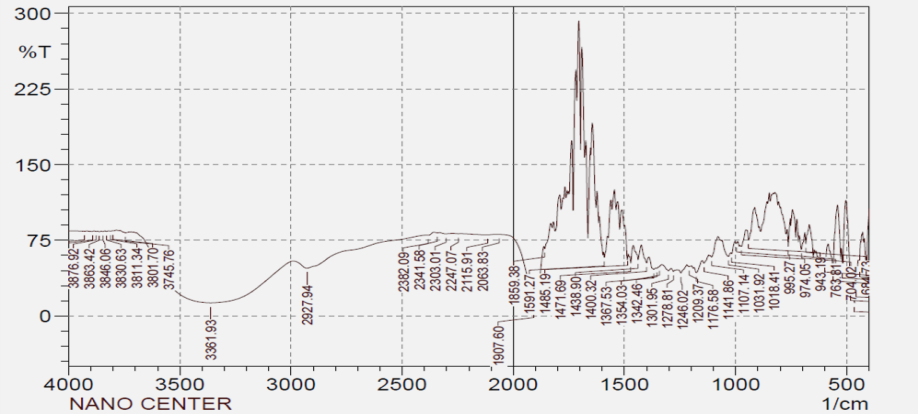

Figure (5): FTIR chart of biosynthesized Ag-Nps

Nitrogen atom may be involved in the binding of AgNPs, resulting in an absorbance decrease. Both N–H and O–H bonds might have different mechanisms when absorbing AgNPs and lead ions. The characteristic amide C-O stretching vibration was observed at 1617–1618 cm^{-1}. This band was also affected, shifting its wave number to 1630– 1632 cm^{-1} [22].

3.4 Nitrate reductase genes amplification

The results showed that *napA1* gene found in 100% of testing isolates and *napA2* gene found in 98 % of them, and amplified size of *napA1* gene was 235 bp, while 201 bp for the *napA2* gene as in Figure (6). Nitrate reductase in *E.coli* is a membrane-bound enzyme, this enzyme is the last enzyme in an electron-transport chain enabling *E.coli* to use nitrate instead of oxygen as the terminal electron acceptor, and this

enzyme permit the bacteria to derive a physiological benefit in the presence of low concentrations of oxygen [23].

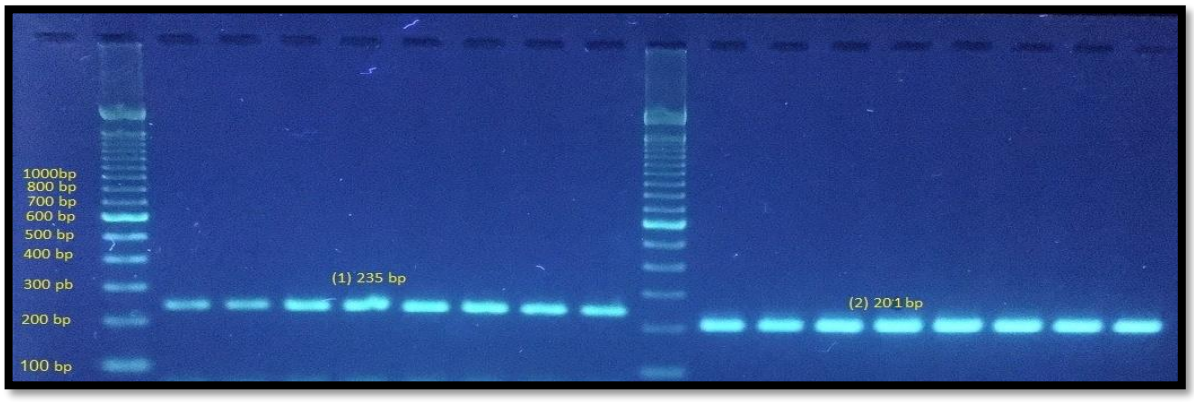

Figure (6): Agarose gel electrophoresis for *napA1*and *napA2* gene. (1.5% agarose, 7V/cm, for 120 min) for *napA1* gene (1) (amplified size 235 bp) and *napA2* gene (2) (amplified size 201 bp) compared with (1000bp) DNA ladder.

These results were in agreement with [24] possessed *napA* gene, which reflects a release of periplasmic nitrate reductase enzyme, and ref. [12] showed that *napA* gene found in 100% of *P.aeruginosa* isolates. Studies have shown that in all the organisms that can synthesize silver nanoparticles, nitrate reductase might be an integral part [25]. The two NAPs genes present may reflect an adaptation of *E.coli* to deal with the different environmental conditions [26].

4. Conclusion

In this study silver nanoparticles were biologically synthesized using *E. coli* isolates which confirmed by using CHROM agar and VITEK-2 compact system. Synthesis of Ag-Nps optimized on 37ºC, pH 9.0 and 10 mM of solid $AgNO_3$. Biosynthesis of Ag-NPs by *E.coli* culture supernatant was confirmed by changes from light yellow to dark brown after 24h incubation. Ag-NPs size ranged from (10.3 to 86.0) nm, and the mean size is 40.2 nm with spherical particles by SEM. Nitrate reductase genes amplified and showed that *napA1* gene found in 100%, while *napA2* gene in 98 % of tested isolates.

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