

# Extracellular biosynthesis of silver nanoparticles by *Haemophilus influenzae* and their antimicrobial activity

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## Abstract :

Biosynthetic methods use either plant extracts or microorganisms have emerged as a simple and viable alternative to physical and chemical methods. Several microorganisms, such as bacteria, fungi and yeasts, have come up as nano factories, synthesizing metal nanoparticles of Ag. Here we report extracellular of silver nanoparticles by *Haemophilus influenzae*. The *Haemophilus influenzae* culture supernatant with silver nitrate (AgNO<sub>3</sub>) carried out in dark and light Intensities, and cultured separately in brain heart infusion broth and tryptic soya broth media. The silver nanoparticles were characterized by Atomic force microscopy. In visual analysis color variation of silver nitrate solution into brown signifies the development of silver nanoparticles. And the antimicroorganism activity of AgNPs produced was evaluated against some local pathogenic microorganism isolates, *Pseudomonas aeruginosa*, *Streptococcus* spp., *Klebsiella* spp, *Staphylococcus aureus*, *Escherichia coli*, *Serratia* spp and *Candida albicans*, using the well diffusion method. Our results indicated that the AgNPs were produced by *Haemophilus influenzae* and the average particle size is different according to type of medium and incubation condition among synthesis of AgNPs, dark and light, The average particle size ranged between 80.05nm-101.15nm. Moreover AgNPs proved excellent antimicrobial activity with inhibition zone ranged between 10mm to 33 mm.

**Keywords:** Silver nanoparticles, *Haemophilus influenzae*, antimicrobial activity, Atomic Force Microscopy.

## INTRODUCTION

The nanoparticles are metal atom with diameter less than 100 nm, highly promising because of their wide selection of applications in commercial products. The metal nanoparticles are synthesized by physical, chemical and biological approaches<sup>1</sup>.

Many of the nanoparticle synthesis methods involve the use of hazardous chemicals, low material conversions and high energy consumption, which pose potential risks to human and to the environment. So, for nanoparticle synthesis without using toxic chemicals a growing need to develop a friendly process. Biosynthetic methods use either microorganisms or plant extracts have occurred as a simple method and alternative to synthetic procedures from chemical and Physical methods. Several microorganisms, such as bacteria, mold, yeast, and plants or plant extracts are already well-documented. However, the exploration of actinomycetes has gained interest in the efficient biological synthesis of nanoparticles<sup>2</sup>.

Research has focused heavily on prokaryotes as a means of synthesizing metallic nanoparticles. Bacteria are a good choice for study, because their abundance in the environment and their ability to adapt to extreme conditions. They are also fast growing, inexpensive to cultivate and easy to manipulate. Growth conditions such as oxygen, temperature and incubation period can be easily controlled<sup>3</sup>. In fact, a number of different species of bacteria are able to reduce metal ions producing metallic nanoparticles with antimicrobial properties<sup>(4-10)</sup>. such as *Bacillus licheniformis*<sup>11</sup>, *Bacillus* spp.<sup>12</sup>, *Klebsiella pneumoniae*; *Escherichia coli*; *Enterobacter cloacae*<sup>13</sup>.

Among nanomaterials, Silver nanoparticles play an important role in the field of biology and medicine due to their attractive physicochemical Properties. Silver nanoparticles are reported to possess antifungal, anti-inflammatory, antiviral, antiangiogenesis and antiplatelet activity<sup>8</sup>

*Haemophilus influenzae* is a small gram-negative coccobacillus, facultatively anaerobic bacteria belonging to the Pasteurellaceae family. pathogenic bacterium responsible for a variety of infections in humans, including respiratory tract infection, septicemia, bronchitis, pneumonia, and acute otitis media respiratory tract pathogen<sup>14</sup>.

In the present investigation, we examined extracellular biosynthesis of silver nanoparticles (AgNPs) from the bacterial isolate *Haemophilus influenzae* used two media, brain heart infusion broth and tryptic soya broth and incubation in light and dark condition. The antimicrobial activity of synthesized silver nanoparticles was also against different microorganisms.

## MATERIAL AND METHOD

### Isolates

All isolates obtained from the Mustansiriyah university / College of Science / Department of Biology, Isolates were primarily identified in our laboratory, then were identified using the Vitek 2 GN ID and VITEK 2 system (Biomerieux) in Al-Kindy Educational Hospital. *Haemophilus influenzae* isolate was used in the present study to test their ability to biosynthesize of silver nanoparticles, while The microbes selected for the antimicrobial activity were *Pseudomonas aeruginosa*, *Streptococcus* spp., *Klebsiella* spp, *Staphylococcus aureus*, *Escherichia coli*, *Serratia* spp and *Candida albicans*. The bacterial isolates were maintained on nutrient agar. While yeast (*Candida albicans*) was maintained on Sabouraud dextrose agar (SDA).

### Synthesis of silver nanoparticles

*Haemophilus influenzae* isolate was cultured separately in brain heart infusion broth (BHIB) and tryptic soya broth (TSB) media to produce the biomass for the synthesis of AgNPs, cultures were incubated at 37°C for 24 hours, The interactions were carried out in dark and light. After 24 hours of growth centrifuged at 1000 rpm for 10 minutes. The supernatants were pale yellow in appearance. To 5 ml of each sample supernatant taken in a test tube, 1 mg of AgNO<sub>3</sub> was added in the laboratory under ambient conditions<sup>15</sup>. A brown mass gets deposited at the bottom of each test tube after 24h. Control was run along with experimental test tubes (without adding AgNO<sub>3</sub>).

### Characterization of Silver Nanoparticles.

The detection of AgNPs was primarily carried out by visual observation of color change of the bacterial filtrate after treatment with silver nitrate. The appearance of dark brown color of bacterial cell filtrate indicates the formation of SNPs. Further, AgNPs was characterized with Atomic force microscopy (AFM)

Analysis, Atomic Force Microscopy was used to obtain the surface topography and the average particle size. AFM image was taken using XE-100 AFM from Park Systems. The aqueous AgNPs were deposited onto a freshly cleaved mica substrate. The sample aliquot was left for 1 min, then washed with deionized water and left to dry for 15 min. The images were obtained by scanning the mica in the air in non-contact mode<sup>16</sup>.

#### Determination of antimicrobial activity

The antimicrobial activities of the Silver Nanoparticles using *Haemophilus influenzae* were evaluated by the well diffusion method against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus* spp, *Klebsiella* spp, *E. coli*, *Serratia* spp and *Candida albicans*.

The test organisms were suspended in saline solution and adjusted to match 0.5 McFarland's Standard. Then cultured in Muller-Hinton Agar plate (Hi Media, Mumbai, India) by using a sterile swab. The plates were allowed to dry and a sterile cork borer with diameter of 6.0mm was used to bore two wells in each agar plate. A 50 $\mu$ L volume of Silver Nanoparticles was applied by micropipette in the wells into Muller-Hinton Agar plate. The bacterial filtrate with out AgNO<sub>3</sub> served as control. The plates were allowed to stand for 1h or more for diffusion to take place and then incubated at 37°C for 24hrs. Antimicrobial activity was determined by measuring the of inhibition zone around each well For each extract in millimeter using ruler , three replicate trials were conducted against each organism<sup>17</sup>.

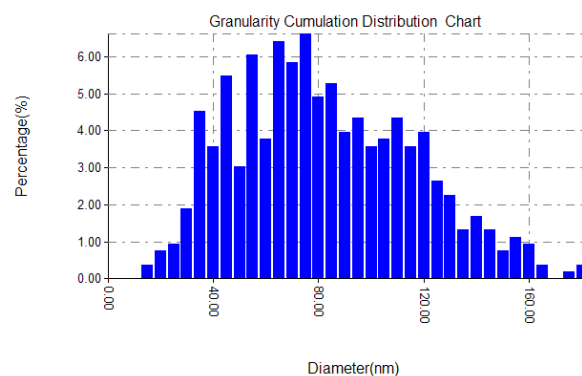
#### RESULTS AND DISCUSSION

In our study, the *Haemophilus influenzae* showed extracellular biosynthesis of AgNPs. The color change was observed upon mixing the *Haemophilus influenzae* culture supernatant with silver nitrate carried out in dark and light Intensities. And cultured separately in brain heart infusion broth and tryptic soya broth media , this was observed by the visual observations of the changes in color of silver nitrate solution from pale yellow to brown color of bacterial supernatant due to excitation of surface plasmon after treatment with silver nitrate indicates the formation of AgNPs. Previous studies have showed that many bacterial species, such as *Lactobacillus* spp., *Pediococcus pentosaceus*, *Enterococcus faecium* and *Lactococcus garvieae*<sup>18</sup>. *Sphingomonas paucimolis*, *Serratia* spp. and *Pseudomonas aeruginosa* are most efficient at synthesis AgNPs extracellularly<sup>17</sup>.

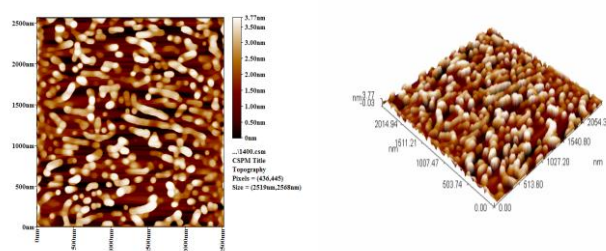
This extracellular production of NPs is a more desirable not only because of the simplicity of purification, but also due to the increased production rate<sup>19</sup>.

A two-step process of AgNP formation was proposed. First, the Ag ions were accumulated at the cell wall via absorption and then subsequent reduction of those ions formed the metallic nanoparticles. Also advocate that the cell wall may act as a capping agent for the nanoparticles, which keeps them stable by preventing aggregation and showed that by increasing the pH of the medium, the reduction rate of the nanoparticles increased<sup>18</sup>.

Atomic force microscopy (AFM) analyses were conducted to characterize the biosynthesize AgNPs by *Haemophilus influenzae* the. (Fig. 1,2,3, and4) views the AgNPs both in surface and three – dimensional view . The shape of AgNPs obtained from *Haemophilus influenzae* isolate are mostly spherical and the average particle size is different according to type of medium, Brain heart infusion broth (BHIB) and Tryptic soy broth (TSB) and incubation condition among synthesis of AgNPs, dark (D) and light (L). The average particle size In BHIBL is 80.05 nm but inBHIBD 97.75 is nm, While in TSBL is 95.54 nm and in TSBD is 101.15. nm.

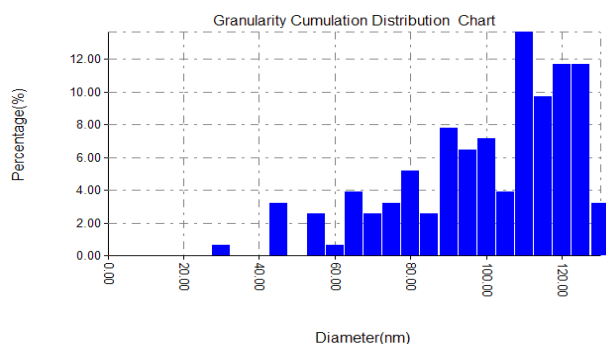


A

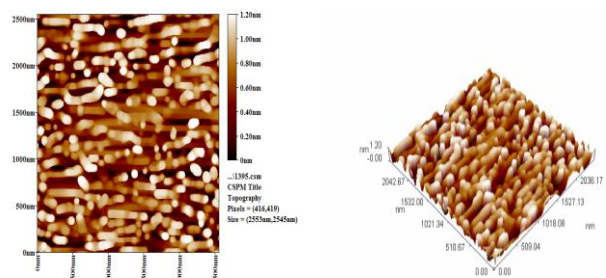


B

**Figure. 1: Atomic Force Microscopy image of AgNPs synthesized by, *Haemophilus influenzae* In BHIBL -A- Diameter percentage, -B- Surface and three Dimensional views.**

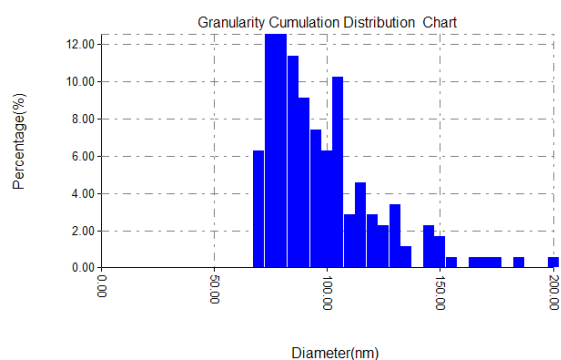


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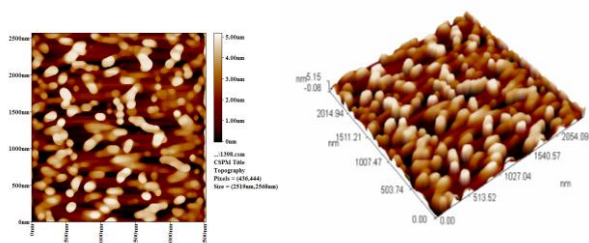


B

**Figure. 2: Atomic Force Microscopy image of AgNPs synthesized by *Haemophilus influenzae*, in BHIBD-A- Diameter percentage, -B- Surface and three Dimensional views.**

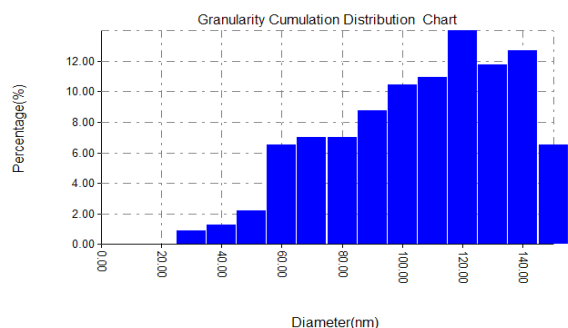


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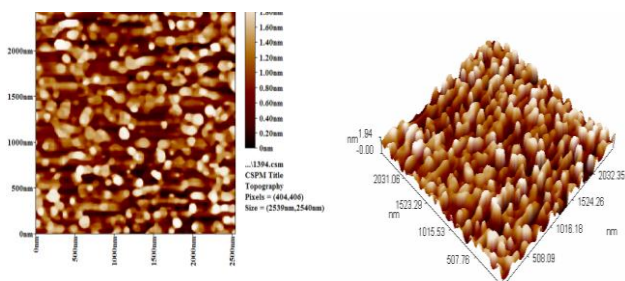


B

**Figure. 3: Atomic Force Microscopy image of AgNPs synthesized by, *Haemophilus influenzae* in TSBL -A- Diameter percentage, -B- Surface and three Dimensional views.**



A



B

**Figure.4: Atomic Force Microscopy image of AgNPs synthesized by *Haemophilus influenzae*, TSBD -A- Diameter percentage, -B- Surface and three Dimensional views.**

This study is the first report of synthesis of AgNPs by *Haemophilus influenzae*, while Various reports provided evidence of extracellular synthesis of AgNPs by some local clinical isolates. The authors reported well distributed spherical shaped AgNPs with sizes 91.74nm, 93.39n m and 93.55n m. By *Sphingomonas paucimolis*, *Serratia* sp. and *Pseudomonas aeruginosa* respectively<sup>17</sup>. Other reports showed that spherical shaped AgNPs synthesis by : *Enterobacter cloacae* (EC), *Enterobacter sakasaki* (ES), *Klebsiella pneumoniae* (KP), *Proteus mirabilis* (PM) and *Raoultella terrigena* (RT). With the size from 79.45 to 102.25nm<sup>20</sup>.

In a research conducted by spherical AgNPs were produced by *Escherichia hermannii* and *Citrobacter sedlakii* with average particle size 4-12 NM and 4-15nm respectively<sup>21</sup>.

To study the effect of different media for production of AgNPs, we grew bacteria on two different media. In the present study, BHIB medium is the best medium for AgNPs synthesis compared with the other medium TSB medium. BHIB medium may raise the extracellular nitrate reductase production and hence increase the production of SNPs. Which may be due to the secretion of nitrate reductase, an enzyme responsible for reduction of silver ions<sup>22</sup>. Moreover production of AgNPs in light is best in both media (BHIB and TSB). There are very few reports, on the photomediated biological production of SNPs. reported that culture filtrate of *Klebsiella pneumoniae* results in the AgNPs production under the irradiation of visible light<sup>23</sup>.

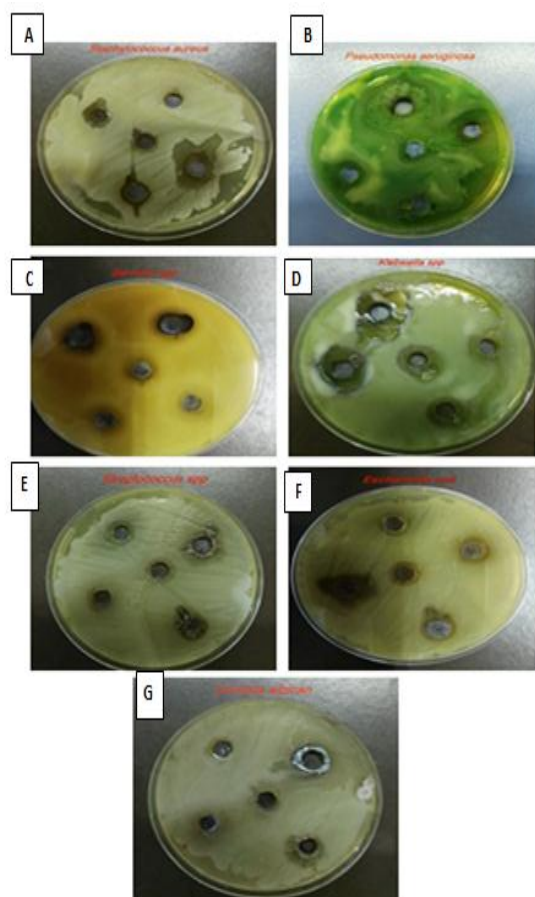
**Antimicrobial assay**

The antimicrobial activity of AgNPs synthesized by *Haemophilus influenzae* was tested against pathogenic bacteria, the gram negative *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* spp, *Serratia* spp and the gram positive bacteria *Staphylococcus aureus*, *Streptococcus* spp and yeast (*Candida albicans*) by using agar well diffusion assay method.

Inhibition zone was determined by measuring the diameter of microbe clearance after 24 h., the maximum activity of silver nanoparticles in case of BHIB in light is found for *Staph. aureus* with 31 mm zone of inhibition, followed by *Pseudo. aeruginosa* 30mm. The diameters of the inhibition zones against *Kleb. spp*, *E. coli*, *Strep. spp* and *Ser. spp* were found to be 24,18,15,15 mm, respectively, while in case of *C. albicans* were 20 mm. (Table 1). The representative zones of inhibition are shown in (Fig.5) On the other hand, when compared with BHIB in dark, we found that the antimicrobial activity of AgNPs was lower against all pathogenic microbes except for *Kleb. spp* ,(33)mm, while *Staph. aureus*, *Pseudo. aeruginosa*, *E. coli*, *Strep. spp*, *Ser. spp* and *C. albicans* were 15,29,15,15,13, and 15mm respectively.

**Table (1): Effect of silver nanoparticles(AgNPs) synthesis by *Haemophilus influenzae* against some pathogenic microbes.**

Microorganisms	Inhibition zone in mm			
	BHIB L	BHIB D	TSB L	TSB D
<i>Staphylococcus aureus</i>	31	15	10	14
<i>Pseudomonas aeruginosa</i>	30	29	15	12
<i>Klebsiella</i> spp	24	33	15	12
<i>E. coli</i>	18	15	13	18
<i>Streptococcus</i> spp	15	15	13	10
<i>Serratia</i> spp	15	13	12	14
<i>Candida albicans</i>	20	15	10	12



**Figure. 5: The inhibition of silver nanoparticles(AgNPs) synthesized by *Haemophilus influenzae* against some pathogenic microbe ,showing the antimicrobial activity against A: *Staphylococcus aureus* ,B:*Pseudomonas aeruginosa* , C: *Serratia* sp, D:*Klebsiella* spp, E:*Streptococcus* spp , F :*Escherichia coli*, and G: *Candida albicans*.**

Moreover, when used TSB medium and incubated in light found that the maximum activity of AgNPs for *Pseudo. aeruginosa* and *Kleb. spp* with 15 mm zone of inhibition, followed by *E. coli* and *Strep. spp* with 13mm. The diameters of the inhibition zones against *Ser. spp* was found to be 12 mm, while in case of *C. albicans* and *Staph. aureus* were 10 mm see Table 1 and Fig. 5. While, when compared with the TSB in dark, we shows that the antimicrobial activity of AgNPs was lower against *Pseudo. aeruginosa* ,*Kleb.spp* and *Strep. spp* with inhibition zone 12,12 and 10mm respectively. While increase against *Staph. aureus*, *E.coli*, *Ser. spp* and *C. albicans* with inhibition zone 14m18,14 and 12 respectively.

Among nanomaterials, AgNPs play an important role in the field of biology and medicine due to their charming physiochemical properties. Silver nanoparticles are reported to possess anti-fungal, anti-inflammatory, anti-viral, anti-angiogenesis and antiplatelet activity<sup>8</sup>.

An interesting study by Ajah *et al*(2016 ) focused on the synthesis of AgNPs by *Sphingomonas paucimolis*, *Serratia Spp* and *Pseudomonas aeruginosa* and there antimicrobial activity against different microorganism(*Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pyogens* and *Candida albicans* ). AgNPs have the highest antibacterial and antifungal efficacy against all the investigated microorganisms<sup>17</sup>.

Dorau *et al.* (2004) reported that the AgNPs cause cell lysis of microorganisms by formation of insoluble compounds that

inactivation of sulfhydryl groups in the cell wall and disarray of membrane bound enzymes and lipids<sup>24</sup>. Also other studies reported that the process of AgNPs may involve the binding of AgNP to external proteins to make pores, or interfering with DNA replication or formation reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anions, and hydroxyl radicals<sup>(25-27)</sup>.

Few contributions were made regarding the effect of cultural and physical conditions on the biosynthesis of AgNPs. The synthesis of AgNPs at the nano-range is still a challenge. Narayanan *et al.*(2008) reported that, for synthesis of AgNPs , it is necessary to optimize the cultural conditions like pH, light intensity and temperature ,In order to increase the yield and the shelf-life (stability) of AgNPs with minimum exploitation<sup>28</sup>.

## CONCLUSION

The usage of bacteria is the good way to the production of Eco-friendly and costs active silver nanoparticles. The rapid and high production of silver nanoparticles was produced by *Haemophilus influenzae* .The present study concludes that *Haemophilus influenzae* can be used as source for synthesis of silver nanoparticles extracellularly. The biosynthetic methods have been known as an alternative to chemical and physical synthesis, due to this biosynthetic method is frugal, ecofriendly, and cost-effective. The present work exhibited an efficient and low-cost biological way to production the metal nanoparticles and provided helpful insight into the development of new antimicrobial agents with the synergistic increase of the antimicrobial mechanism against pathogenic microorganisms. Biosynthesized silver nanoparticles have potent antimicrobial activity contra *Pseudomonas aeruginosa*, *Streptococcus* spp., *Klebsiella* spp, *Staphylococcus aureus*, *Escherichia coli*, *Serratia* spp and *Candida albicans*.

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