Bio-synthesis of silver nanoparticles from bacteria *Klebsiella pneumonia*: Their characterization and antibacterial studies.

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ABSTRACT

Epidemics of infectious acute diseases are caused by bacteria that cause various diseases and the increase of antibiotic resistance, which has encouraged drug companies and researchers to search for new antibacterial agents. The aim of this study, involved the creation, characterization and antibacterial studies of silver nanoparticles by using bacterial culture supernatant of bacteria *Klebsiella pneumonia* for the production. We used an eco-friendly extracellular bio-synthetic method for the production of the silver nanoparticles. The biosynthesis SNPs solution were initially categorized by several techniques, the UV-visible spectrophotometric record absorbance a powerful peak at 432 nm, analysis (FTIR) Fourier Transform Infrared and (SEM) Scanning Electron Microscope. The SNPs solution showed anti-microbial activity against different types of pathogenic bacteria that used in the present study: Gram negative (*Pseudomonas aeruginosa, Escherichia coli*), Gram positive: (*Staphylococcus aureus, B. cereus*).

1. INTRODUCTION

Nano-technology is high-speed science that related to other sciences like Physics, Chemistry, Biology, Engineering and other sciences (Islam and Miyazaki, 2009). Its deals with Nano-particles sized 0.1 to 100 nm; also these compounds displayed different properties like electrical conductance, magnetism, chemical reactivity, physical strength, thermal conductivity, chemical stability, and optical effects, from huge compounds due to their small size (Ju-Nam, 2008). The tiny sizes, make it a technology of highly importance in multiple uses (electronic engineering, communications), moreover, this technology has been accommodation in the field of cosmetics,
medicine, environmental remediation, renewable energies , and biomedical devices Chaudhuri & Paria , 2012) Nano-particles considered an alternative to antibiotics and may have high potential to end the problem of the development of multidrug resistance in bacteria. One of the superlative approaches of synthesis is the biological method by using plant ,fungus , or bacteria to produce Nano-particles (Parveen et al. , 2017). Silver nanoparticles (AgNPs) are very important among the most used metallic nanoparticles Because of its exclusive chemical-physical properties as it has been used to control and prevention of numerous infections through their interaction of bacterial membranes, leads to the death of the pathogen (Salvioni et al., 2017). Several studies have proven that AgNPs activity is highly dependent on the size of tiny Nano-particles that have a higher capability to infiltrate into bacteria (Wu et al., 2014). Silver is a white soft shiny material. Metallic silver has no water solubility, but its metallic salts such as Silver chloride and AgNO3 are water soluble. Metallic silver have become the effort of many researchers in biomedical field, it discovered about 100 years ago, and used in the treatment of infections before the discovery of penicillin in 1928, silver Nano-particles are produced by transforming silver metal into silver of smaller size, that is very effective against microbial infections (Brett , 2006).

The effect of silver nano-particles is deadly and inhibitory to bacteria as it can be used as a treatment for numerous infectious diseases (Afreen et al., 2011). The accumulation of silver Nano-particles on the cellular membrane results in gaps in the integrity of the membrane layers, thus increase the permeability and finally death of the bacteria (Rajeshkumar and Malarkodi ., 2014). AgNP are found in silver metallic form, and are strongly attached together ,when the AgNPs range from 10 to 100 nanometers, they are take dissimilar forms (flat spherical, or irregular) depending on how they are prepared, Silver, is a natural element, does not accumulate in the body, hypoallergenic, non-toxic to cause harm and is harmless to the environment (Parveen et al., 2017).

There are numerous methods, including chemical, biological, and physical, for the synthesis of nano-particles , there are cons and pros to each of these methods with some difficulties such as the size of the nano-particles and cost (Tran et al., 2013). Biological synthesis considered the most common friendly method to the environment, it considered as a possible eco-friendly alternatives to the physical or chemical production; because both methods are coasty (Parashar et al., 2009). In addition, the chemical synthesis deals with toxic chemicals, which poses biological and environmental hazard, therefore; living cells of bacteria, fungi and plants are preferred by researchers to produce silver nano-particles, there has been interest in using eco-friendly methods of manufacturing nano-particles without making or using materials risky to the environment and human health. (Mohandas , 2017)

Bacteria is one of the most important biological sources for producing nanoparticles because of its advantages such as its secretion of extracellular enzymes which works to reduce the metal
ions and thus produce nanoparticles, their rapid growth, simple of culture and preservation in vitro , Therefore, their use in nanotechnology is rather inexpensive and nano production can be controlled by manipulating several conditions including temperature, pH, the concentration of metal ions, and reaction time making them ideal for use in biosynthesis to produce nanoparticles (Hamid & Mohsen. 2019).

2- MATERIALS AND METHODS

Culturing the microbe :

Bacteria *Klebsiella pneumonia* were cultured on nutrient broth and incubated at 37°C for 24 hours to prepare the bacterial suspension, then transported the bacterial suspension to a tube 10 ml, and were centrifuged at (6000 rpm, 25 minutes). the supernatant remove to new tube while the pellet that found in the bottom of the tube is discarded (Singh *et al.*., 2013).

**Bio- synthesized silver nanoparticles :**

Biological methods have received extensive attention to synthesis nanoparticle , Various biological resources available for green synthesis of silver nanoparticles are bacteria (Siddiqi and Husen., 2016). 100 milliliters of nutrient broth was prepared inoculated with the bacteria *Klebsiella pneumonia* broth media in 250 ml conical flask , then flasks that containing media were incubated at 37 °C, for 1-3 days in incubator after that, the culture solution was centrifuged at 13500 rpm. for 10 min, then the cells pellets were discarded and the supernatant was collected for use in the biosynthesis of silver nanoparticles. The supernatant (100 ml) was taken into a clean 250 ml conical flask and (100 ml) of ( 1, 2, 4mM ) silver nitrate was added to the supernatant then the mixture was incubated in the shaking incubator at 100 rpm, 37° C for 24 hours in dark condition to avoid of AgNO3 oxidation for the biosynthesized SNPs, the color change was observed visually and photographs were taken. (Song and Kim , 2009).

**Characterization of biosynthesized SNPs**

Extracellular bio-reduced silver ions by the culture supernatant of the bacteria *Klebsiella pneumonia* were preliminarily examined by:

**UV-Visible spectroscopy analysis**

Used for the observation of the brown color of the mixture with the silver nitrate solution, which helps us to character the nano-silver particles. The spectra of the UV spectroscopy was recorded by taking (3 ml) of a sample of the reaction solution at different conditions using UV- Vis spectrophotometer (200-800nm). (Chul *et al.*., 2012)

**Fourier Transform Infrared Spectroscopy (FTIR)**

Fourier transformed infrared (FTIR) spectrum of the sample was recorded by Fourier transform infrared (Nicolet 6700 FT-IR, Thermo Scientific) spectrophotometer. The FTIR
spectrum ranged from 4000 to 450 cm−1 at a resolution of 4 cm−1 by making a KBr pellet with AgNPs (Martak et al., 2019)

**Scanning electron microscope (SEM)**

SEM was used for characterization the morphological and size of nanoparticles in electron microscope unit, Preparation of slides by adding a small drop of suspension of biosynthesis nanoparticles on slides, and left to dry and then analyzed by (SEM), The microscope operated at an accelerated voltage at 5-10 KV and different magnification, low vacuum, a spot size 4 and working distances 5-10mm (Li et al., 2010).

**Effect of some physic-chemical condition**

Different parameters were taken, such as supernatant size, silver nitrate concentration, temperature, pH. the experiment was conducted in three replicates and the results have been monitored using the UV-Visible spectrophotometer

**Effect of supernatant size**

Examined different volumes of bacteria culture supernatant (2 ml, 4 ml, 8 ml) in the mixture solution to obtain the optimum supernatant size of the substrate for SNPs production. the optimum supernatant size was detected on the basis of the characterized by UV Visible spectrophotometer (Hulkoti and Taranath., 2015).

**Effect of concentration of silver nitrate**

Designed the pilot experiment by examining three different concentrations of silver nitrate (1mM, 2 mM, 4mM) in the reaction solutions to Discovery of the optimal concentration which is the most important factors that make the interaction more economical and effective were the substrate that can be converted into the final product. the optimal concentration change in color and was detected on the basis of the change in color and characterized by UV Visible spectrophotometer (Vikas et al., 2015).

**Effect of pH**

Experiment was designed to investigate the optimal pH for the synthesis of nanoparticle particles, PH (4, 6,7,8,10) was set in plastic screw cap tubes that containing 4 ml. of supernatant that prepared in (3.2.5.3) and(1mM.) of silver nitrate all the tubes were incubated at 25° C for 24 hrs. and 100 rpm. in a shaking incubator, Acetic acid was used to adjust the pH < 7, While NH3 to adjust the pH <7, the optimum pH was detected based on the color change and characterized by UV Visible spectrophotometer. (Patra and Baek, 2014).
Effect of temperature

Temperature is an essential factor affecting on AgNPs production so the pilot experiment was designed to detect the optimum temperature for AgNPs bio-synthesis, tubes were prepared that contained 4 mL of the supernatant at the optimum concentration (1mM) of the substrate at gradient temperatures (30,60, 90°C) in heating mantle until color change until color change the optimal temperature was detected based on the color change and characterized by UV Visible spectrophotometer(Husam and kredy., 2018).

Purification of biosynthesized silver nanoparticles

Silver colloids were centrifuged at (6000 rpm, 25 minutes) after optimization of synthesis conditions , the supernatant was discarded, replaced with de ionized distilled water, and washed three times with millipore filter to remove the residue of supernatant, while the pellet that found in the bottom of the tube is dried at 40°C (18-24 h) and collected dried powder gently and stored for other tests (Gurunathan et al., 2009).

Determination of antimicrobial activity of SNPs :

The SNPs synthesized from Klebsiella pneumonia were tested for antimicrobial activity by well-diffusion method against pathogenic organisms such as Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa and Bacillic Ceruis Wells of 6-mm diameter were made on Müller-Hinton agar plates using gel puncture. Each strain was swabbed uniformly onto the individual plates using sterile cotton swabs. 100μl from 150 μg/ml concentration of AgNPs was distributed into one of the wells, and bacteria culture supernatant is placed in the other well as a control and the third end of the petri dish in which placed a loaded nano silver particle was done by depositing every 10 tablets of filter paper in 5 ml of 150 μg/ml SNPs for two-days and incubated at the same degree and measured different levels of inhibition zones.( Sanchooli et al., 2018)

3-Results and Discussion

Extracellular biosynthesis of SNPs by K. pneumoniae

Visual Examination

K. pneumoniae was inoculated in nutrient broth, 4mM of AgNO3 added to the bacteria culture supernatant (BCS), then incubation 24hr in dark condition, the reduction will causes the color changes in mixture of bacterial supernatant from light yellow to brown color ,then the color becomes darker by increasing the incubation period, this refers to the ability of SNPs biosynthesis through the bio-reduction of Ag ions and the formation of , while observed in the control sample no change in color that containing bacteria supernatant without AgNO3 when incubated in the same conditions. Figure (1)
Fig 1: Show (A) Bacteria Culture Supernatant without AgNO₃. (B) Culture Supernatant of bacteria Mixture with 4mM AgNO₃.

**UV-Visible spectrometry**

UV-Visible spectrometry is the technique which is used in visualization of nano-silver particles changes in the medium, because the ability of SNPs to absorb light in the visible region due to the surface plasmon resonance (SPR) phenomenon, when analyzed at room temperature by UV-Vis spectrophotometer and the absorbance of the sample was read at the wave lengths of 200-800 nm,. Results after 24 hours showed the measurement of the visible absorption spectrum of ultraviolet rays different absorption peaks at limited wave lengths and the absorption intensity gradually increased to the highest absorption indicates a continuous decrease in silver nitrate lead to an increase in the concentration of silver nano-particles indicates their formation in the reaction mixture. Figure(2). Peak of Ag NPs absorption was adjusted at 432nm., while no absorption peaks at the wave lengths mentioned in bacteria supernatant that untreated with AgNO₃ used as a control agent. (Kirubha and Alagumuthu, 2013). The absorption range may be attributed to residues of tryptophan and tyrosine present in the protein. This indicates secretion of some protein components into the medium from the bacterial biomass Which may be the main factor in the reduction of the metal ions in the form of nano-particles. Consequently the proteins also may bind to the nano-particles enhancing the stability of absorption (Ponarulselvam et al., 2012).

Fig (2): UV-Visible spectrometry analysis of biosynthesized silver nano-particles
Scanning Electron Microscope
SEM obtainable more vision to search for the size and morphology details of the SNPs. From the results, the size of SEM was ranged from 26.84 to 44.42 nm. Individual SNPs and some assembled spherical particles Figure (3). Nanoparticles conglomerate in the process of drying samples have been prepared for examination. In general, the characteristics of monodispersion, shape, size of particles were highly dependent on the function of these particles, the obtained data from SEM analysis were very similar to the findings of (El-Shanshoury et al., 2011) whom reported bio-synthesis of SNPs using culture supernatant of isolated Bacillus sp. Also, Mono-dispersed SNPs in the range of 50 to 120 nm was extra-cellular synthesized by Vithiya et al., (2014).

Fig (3) : SEM electron micrograph of the SNPs biosynthesized by the bacteria k. pneumonia. Power magnification 79 959 x.

Fourier Transform Infrared Spectroscopy (FTIR)
FT-IR measurements were performed in this study to identify the potential biomolecules in the reaction mixture. the FTIR spectra of bio-fabrication of SNPs presented four distinct peaks measuring (3332.78, 2115.35, 1635.60 and 1096.92, cm⁻¹) Figure (4).
The peak 3332.78 cm⁻¹ is ascribed to the stretching vibration of OH bond of alcohol, phenols, is the characteristic band of hydrogen bonded group of OH that may be due to the formation of nano-particles from the aqueous phase. (Das et al., 2013) the peak at 2115.35 cm⁻¹ might be due to the C-H stretch of the methylene groups of protein and to N-H stretching of amine salt. This result is probably related to the modification of the electronic environment of groups the methyne and methylene influenced by the adjacent carbonyl and the silver nanoparticles. (Fayaz et al., 2009). 1635.60 cm⁻¹ is refered to carbonyl groups (C=O) of the amino acid residues and 1096.92 cm⁻¹ (C–O) stretching of alcohols, esters, carboxylic acids and C–N stretching of aliphatic amines. From these results we can conclude that the presence of protein in the supernatant acts as capping agent for stabilization, and can bind to SNPs, either through free cysteine or amine groups in proteins, this result similarity with Al-Harbi et al., (2014), who detected SNPs produced by biosynthesis extracelluar of silver nano-particles from Bacterium Proteus mirabilis by FT-IR.
Physic-Chemical conditions specific to the biosynthesized SNPs supernatant Volume

In the present study, three different volumes were used: (2, 4 and 8 ml) the effect of the bacterial culture supernatant volume on the synthesis of SNP at 4mM of silver nitrate was depended. However, 4ml of bacterial culture supernatant exhibited maximum SNPs synthesis, this volume (4 ml.) is depended in the first time in this study as a minimum volume that can obtain SNPs from 4mM silver nitrate. This concentration was favorable in this study; because it gave the smallest nanoparticle size that have the optimum ability to penetrate the bacterial envelop and destroy it due to their size, because the smallest particles easily penetrate microorganisms and have greater toxicity effects; however, the larger ones cause less toxicity (Azam et al., 2012).

UV spectra results of present study showed a positive correlation between the increasing intensity of surface plasmon absorbance and the volume of bacterial culture supernatant This result agreed with Divya et al. (2016). Who noticed that the absorbance increased from 307 to 309nm. when the size of the culture supernatant increased from (20-30 ml) for bacteria Escherichia coli.

Temperature

The temperature could play important role in particle formation, shape and size especially for silver nanoparticles therefore, in this study, Various temperatures degree were used (30°C, 60°C, 90°C) to reach the optimum forms of nanoparticles. During color change and measurements of absorption by UV radiation with peak at 423 nm the results showed that the optimum temperature for the biological reduction of Ag was 90°C. Figure(4-13), this temperature (90°C) allowed the particles to be created at a faster rate. This result clarify the effect of temperature on the biosynthesis of nanoparticles, the reaction solution containing the bacterial supernatant and the silver nitrate was change the color from pale yellow to brown within 24 h, the
color becomes darker with increasing temperature Figure (5). Most reports revealed that the reaction temperature affects the size of the nano-particles, as the temperature increases the particles size becomes smaller, and this is proven by Fayaz et al. (2009) An increase in the size of nanoparticles when the reaction temperature is low, while an increase in temperature reduces the size of nanoparticles

![Image](image_url)

Figure (5 ).showing bacterial culture supernatant of *k.pneumona* At multiple temperatures: (A) 30 °C, (B) 60 °C, (C)90 °C

**pH**

In present study the synthesis of silver nano-particles is faster under alkaline conditions as compared to acidic, the synthesis of silver nano-particles increased as the pH towards the alkaline area where it reaches the maximum at pH 10, the appearance of the level absorption ranges at pH 4 and 6 indicates that there was no synthesis of SNPs due to they damaged by the acidity, Also, in the acidic milieu the biosynthesis take a longer time to change from yellow to dark brown. Moreover, high PH leads to make the nanoparticles to tend to fusion resulting the aggregation of these particles(Gurunathan et al., 2009).

The highest peak was 425 at pH 10, alkaline pH condition facilitated the reduction and stabilizing capacity of nitrate reductase enzyme, catalyzing the synthesis which probably activated and become more alkaline, and this may be the reason for increase synthesis of AgNPs and elevation of absorbance that observed at higher pH values (Prasad et al., 2010).

**AgNO3 concentration**

In this study, the biological factors and the concentration of silver nitrates have a significant and visible effect on the biological synthesis of SNPs and this is illustrated by visual examination of color alteration from pale yellow to dark brown in the solution and surface plasmon absorbance peaks of SNPs. The mixture prepared with 4mM AgNO3 solution showed surface plasmon absorbance peaks at 421nm, the peak density was increased by increasing the concentration of AgNO3.
(1,2,4) mM, which indicates a faster rate of bioreduction with increased concentration of AgNO₃, the biosynthesis of silver nano-particles depends also on the dealings of the other factors (Lee and El. sayed., 2006).

The results of our study show that the optimal concentration for silver bio-reduction was 4mM as as shown, due to the communication between the ability of proteins in the reaction medium and enzymes reductase in addition to the Ag⁺ concentration to to obtain the equilibrium suitable very important for the biosynthesis silver nano-particles (El-Rafie et al., 2012).

**Silver Nano-particles as Antimicrobial agent**

Development of drug resistance in pathogenic microorganisms and cause advanced infectious diseases is a very worrying threat cause increased the morbidity and mortality by infection microbial Therefore, there is necessary to identify new antimicrobial agents and discover new strategies to evaluated the next generation of factors or drugs to control diseases infections. (Fair and Tor.,2014).

The use of nano-particles is increasing in the current century because they possess specific chemical and physical properties. Mineral nanoparticles are very important because they show good antibacterial properties that appear as the current interest in researchers because of the increased resistance to many microbial strains to anti-microbial agents. (Hiramatsu et al., 2014). Silver NPs have been tested in various fields of biological sciences, medicine and the world of drug delivery its conceded very important because they are nontoxic to the human and animal body when used at low and specific concentrations it’s have a wide and effective effect as antimicrobials agent causes inhibition of both gram (+) and gram (-) (Willing et al.,2018). Three concentrations (50, 100, 150) µg/ml of SNPs were tested against the bacteria in the present study by using well diffusion method; to identify the concentration that reveal broader zone of inhibition, as shown in figure (6). The concentration 150 µg/ml gave broader zone of inhibition, while the concentrations (50, 100) µg/ml of SNPs are less inhibition zone, this result may regarded to the fact that increase the concentration of SNPs leading to increase the antibacterial efficacy. Also, saturated filter paper in silver nanoparticles at 150 µg/ml concentration was used (Yamamoto et al., 2004). This study revealed that there is significant increase in the inhibition efficacy (P<0.05), when we used the concentration 150 of SNPs to all bacteria that tested in the present study.
Fig(6): Three concentrations (50, 100, 150 µg/ml) of SNPs against the bacteria under study, laden disc, and BCS as control.

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