Antibacterial Activity of Silver Nanoparticles Synthesized by Enterococcus Faecium Against Some Pathogenic Bacteria

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Abstract

The biological technique is widely used for producing nanoparticles compared with other technique and it depends on using living organisms. Local Isolate of Enterococcus faecium was used in this study to synthesize silver nanoparticles. Silver nitrate (AgNO3) was used as precursor for biosynthesis of silver nanoparticles by Enterococcus faecium. the primary characterization of the synthesized nanosilver by Scanning Electron Microscopy, Transmission Electron Microscope, X-Ray Diffraction Dynamic Light Scattering Technique,and FT-IR Technique. The antimicrobial activity of synthesized nanosilver against Four local isolates : Staphylococcus epidermidis , Streptococcus mutans , Esherichia coli and Klebsiella pneumoniae , were estimated by using well diffusion method . The results showed that nanoparticles have a higher effect on Klebseilla pneumoniae as multi drug resistant bacteria compared to other isolates used in this study , and therefore nanoparticles can be a great prespective to resolve the growing rsistance antibiotics problematic .

Keywords: Silver Nanoparticles; Enterococcus faecium; Pathogenic Bacteria

1. Introduction

Enterococcus faecium is a nonpathogenic lactic acid bacterium with applications in food engineering and also protect mankind from various diseases due to their antimicrobial activity (Santos et al., 2020: Dowdell et al., 2010). E. faecium produce antimicrobial peptides (Bacteriocines) were found to be able to effectively inhibit the development of many food pathogens (Yerlikaya et al., 2020; Sharma et al.,2020) with safety probiotic properties (Akpinar et al., 2016). They can survive in a variety of habitats such as soil, sewage (willem, 2017), food plants and animals (Graves and Weaver,2010 & Fran zetti et al., 2004) . They are mainly found in the gastrointestinal tract of human, birds and other mammals as commensals microorganism (Mundt ,1986; Martin & Mandt, 1972).

Nanotechnology it is a branch of technology associated with the synthesis, characterization and application of materials in a nanoscale range of 1-100nm (Bayda et al, 2019). The biological technique is widely used for producing nanoparticles compared with other technique and it depends on using living organisms. Microbial synthesis of metal nanoparticle can take place either intracellularly or extracellularly (Li et al ,2011).

Nanoparticles synthesis by Bacteria

Bacteria possess remarkable ability to reduce heavy metal ions and are one of the best Candidates for nanoparticles synthesis. The first synthesis of Ag nanoparticles by bacteria was reported in 2000. Iravani, *et al.*, 2014) used *Pseudomonas stutzeri* AG 259 to synthesize Ag nanoparticles with size less than 200 nm. Silver in all its forms has been historically used as an antimicrobial agent by itself or combined with other technologies (Silva *et al.*,2017). Ag NPs are defined as a nanomaterial with its dimensions in the range of 1- 100 nm. These have shown greater capacity and higher, surface (area tor volume ratio) compared to silver in its bulk form. AgNPs have shown antimicrobial activity against a variety of infectious and pathogenic microorganisms, including multidrug-resistant bacteria (Siddiqi *et al.*,2018 & Marambio-Jones & Hoek, 2010).The enhanced antibacterial activity of Ag at the nanoscale has been most valuable in medical and health care areas where the incorporation of ANPs into hundreds of products has been studied including surgical and food handling tools, clothing cosmetics, dental products, catheters, and dressings (Kulkarni ,2014; Ge *et al.*,2014). The potential of AgNPs as antibiotics Ag NPs related to their various mechanisms of action which attack microorganisms in multiple structures at a time and give them the ability to kill various of bacteria(Cheng *et al.*,2016) .As infections caused by antibiotic resistant microorganisms are a matter of concern. Ag NPs arise as an excellent alternative as they can be applied to prevent infections caused by these microorganisms, decontaminate medical supplies, and even combat infections in course (Betts *et al.*,2016; Natan & Banin 2017). As an antibiotic alternative this application has been broadly studied in recent years with the objective of developing new bactericidal products for decontamination or infection treatments talking

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advantage of the already established knowledge about their efficiency even against multidrug resistant organisms (Lee *et al.*,2019).

2. Material and methods

Bacterial Isolate

Isolate of *Enterococcus faecium* used in this study were isolated as described by (AL-Taee, 2021). They were characterized using *Enterococcus faecium* chrome agar and VITEK 2 compact system technique.

Silver nitrate solution (1 mM) solution was prepared by dissolving 0.34 g. of silver nitrate in 200 ml of distill water. The solution was used as a precursor of silver nanoparticles (Chaudhari *et al.*,2012; Al-Dujaily *et al.*,2017). A purified bacterial isolates of *Enterococcus faecium* have been cultured in 100 ml of nutrient broth, incubated at 37° C for 24 hr. At the end of incubation period, the culture was centrifuge at 9000 rpm/min for 15 min to separate the bacterial cells. The supernatant was collected in a new sterile conical flask, the supernatant was discarded and replaced with deionized distill water and re centrifuged three times at the same speed and time to remove the remaining supernatant .

Synthesis of AgNPs

The ability of bacterial supernatant to produce nanosilver was evaluated by mixing the bacterial supernatant with 1 mM of $AgNO_3$. The color variation was followed up and the appearance of yellow-brown indicates the nanosilver production. Silver nitrate (AgNO3) was used as precursor for biosynthesis of silver nanoparticles by *Enterococcus faecium*. Silver nitrate was added to cell free supernatant of *Enterococcus faecium* which distributed in sterilized tubes and mixed well. After mixing, deposit at bottom of tube which represent collection of nanoparticles then dried in oven at 40 °C for 18-24 hours. The dried powder was collected carefully and stored in sample vials for further analysis (Chaudhari *et al.*,2012 & Sarvamangala *et al.*,2013).

Characterization of Synthesized Nanosilver

The visible spectra analysis was used for the primary characterization of the synthesized nanosilver by DLS analysis, SEM,TEM, XRD and FT-IR .

Scanning Electron Microscopy(SEM)

SEM was used to characterize nanosilver according to their size, morphology, transparent, and surface rough (Palmqvist,2017). The silver nanoparticles were precipitated by centrifugation at 12000 rpm/min and washed with water several times to obtain a pure participated nanosilver particles precipitate and measured with SEM (Xie *et al.*,2017)

Transmission Electron Microscope(TEM)

It was followed up to confirm the characterization of nanosilver by measuring the particles size and determine the shape (Wang,. (1998). Briefly 10 µl of each sample was placed on a carbon - coated grid, and the excess samples were removed using a piece of blotting paper, After drying the sample under un infrared lamp, the electron micrographs were obtained and photographs were obtained using a digital camera (Pourali, 2017)

X-Ray Diffraction (XRD)

This technique depends on the interference between X-ray and the crystalline sample and that the reflected rays will be recorded and converted into numerical values. The hydrodynamic diameter of particles was measured by scattering dynamic light using Scherer's equation:

$\tau = (K*\lambda)/(\beta*\cos\theta)$, where τ is the nanoparticle size , K is the constant Equation 0.9, λ=1.5 represents the wavelength of the x-ray , β is the total width at the top of the beam, θ is the angle of calculation (Tripathi,2014). The sample was prepared by precipitating the nanosilver solution by centrifugation 12000 rpm/min , washing several times with distilled water, and then a suspension was prepared using the ethanol then distilled onto the surface of a glass plate2*2 and continued until the plate was covered with a thick film of nanoscale gold, then it was placed over a heat heater at 80°C and examined by placing the plate in the X-ray diffractometer and the spectrum was recorded within the range $2°\theta(20° - 80°)$.

Dynamic Light Scattering Technique

The particle size ,poly dispersity index nanosilver in the solution were measured by the DLS technique (Barabadi et al., 2014). One milliliter of the isolated gold nanoparticles was analyzed in 3 m/l a plastic cuvette. The dynamic light scattering measures, the speed of particles undergoing Brownian motion and speed of the Brownian motion is influenced by particle size sample viscosity and temperature. Velocity of the Brownian motion is defined by the translational diffusion coefficient (D) ,which can be converted into a particle size using the stokes – Enstein equation :

$dH = \kappa T / 3 \eta \pi D$

Where: dH = hydrodynamic diameter ,k= Bultzmann's constant (1.38X15 23 Nmk-1), T = absolute temperature , η = solvent viscosity (N sm-2), and D= diffusion Coefficient. (Sandhu et al.,2018).

FT-IR Technique

The solutions were dried, at 80°C drying then the samples have been crushed with KBr in a mortar at a ratio of 1:100 . The pressed pellet was recovered with a clip then analyzed in the range of 4000 -400 cm^{-1} at a resolution of approximately 2 cm^{-1}.(Kitching, 2015)

Purification of Nanosilver

The method described by De Souza et al .,(2019) was followed up for the purification of nanosilver . Nanosilver purified by using a centrifuge at 10,000 rpm/ for 20 minutes to get rid of any suspended impurities It was also washed with non-ionized distilled water 2-3 times after getting rid of the water. The

remaining mass was dried at 50°-60°C for three hours and after it was dried completely, they were collected and gently crushed using ceramic mortar until they became powdered. The dry powder was kept in Eppendorf tubes and the tubes were covered with aluminum foil and stored until use in subsequent applications.

Antimicrobial Activity of Synthesized Nanosilver by *E.faecium*

Determine the Inhibition Zone

Four bacterial isolates whose sensitivity to nanosilver were tested, which included Staph.epidermidis , Strep. mutans, E.coli and Klebsiella pneumoniae , all these isolates were obtained from AL-Hakeem Hospital which were confirmed to be diagnosed using the VITEK2 compact system . The antimicrobial activity was estimated using a well diffusion method (Holder and Boyce,1994). All bacterial isolates were refreshed by the incubation of bacterial isolates on nutrient broth for 24hr at 37 °C. Mueller Hinton agar was prepared according to the manufacture recommendation and after sterilization was poured off in petri-dish and left to solidify under a sterilized conditions. Later on, 0.1 ml of bacterial suspension turbidity equal to McFarland tube No. 0.5. It was prepared by diluting the bacterial culture using sterile medium or sterile normal saline streaked on the surface of MHA using sterile swab. Three wells at each culture plate were done using a sterile well cutter (9 mm) by cork borer, 0.1 Microliter of nanosilver for each concentration has been filled the well , left to diffuse at room temperature then the plates were incubated at 37°C for 24hr. The diameter of the inhibition zone of each concentration was measured using a ruler (Fingold and Martin,1982)

3. Results and Discussion

Biosynthesis of Nanosilver by *E.faecium*.

The isolate of E.faecium has been identified and characterized by (AL-Taee,2021) and studying the ability of bacteria to produce ability to synthesize nanosilver. The primary detection of nanosilver by E.faecium was depended on color changes Figure (1). The conversion of extracellular medium color clearly designates that the process of formation of AgNPs is extracellular in nature (Bhainsa and D'Souza, 2006; Shaligram, et al., 2009). The appearance of a brown color in a solution mixture containing the biomass is a clear indication of the formation of silver nanoparticles in the reaction mixture and is due to the excitation of surface plasmon vibrations in the nanoparticles (Ahmad et al., 2003a). Nanosilver have been characterized by different technique involved SEM,TEM,DLS,XRD and FT-IR.

The results have shown that the optical characteristics of particles influenced different bioreductant which refer to the shape and size of particles, so that the variation in the color of colloidal nanosilver synthesized by E. faecium improved that the cell – free extract of E. faecium contains biomolecules responsible for the biofraction of nanosilver

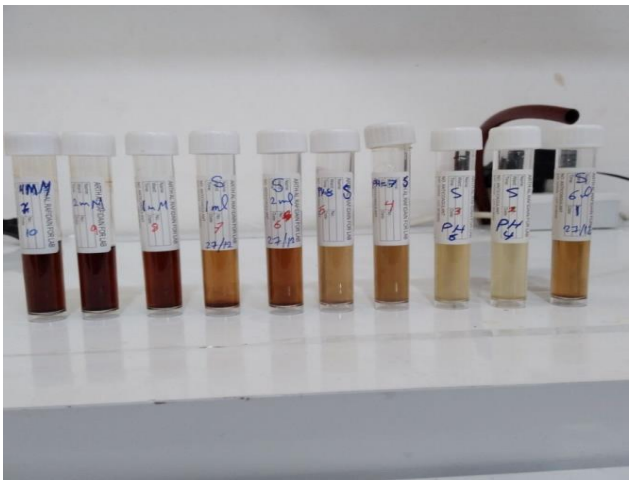


Figure (1): The color variation of synthesized nanosilver from E.faecium

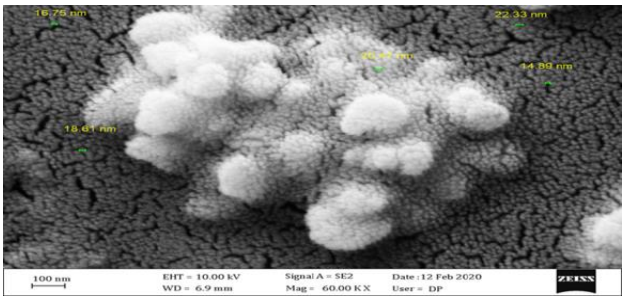
The appearance of color is a clear indication to formation of silver nanoparticles in the reaction mixture due to reduction of Ag+ ions to Ag metal by the reducing agents (amino acid, polysaccharides, proteins , enzymes etc). in the cultural supernatants which were harmless environmentally and complex chemically (kaler et al.,2010; Natarajan et al.,2010; Sreedevi et al.,2015). The ability of bacterial isolate to biosynthesize of nanosilver was detected by visual observation. The ability of cell free extracts of E.faecium to synthesize nanosilver was characterized by the formation of yellow colloidal solution within 4 min. The stability of color was observed after 45 min. The formation of brown color reveals the synthesis of nanosilver (Roshmi et al.,2015).Previous study believed that protein molecules and enzyme, including nitrate reductase act as good regulating agents for biosynthesis of AgNPs using the two microorganism (Al-Dujaily et al, 2017). The color showed by metallic nanoparticles is a result of the coherent excitation of entire free electrons within the conduction band, leading to surface plasmon resonance (SPR) (Sarvamangala et al.,2013). Culture supernatant is found to be the easiest way for the size-controlled synthesis of silver nanoparticles. The environment parameter of the culture supernatant can be maintained and easily modified than the biomass, where the components in the cytoplasm would try to maintain constant environment such as heat shock proteins and require more purification, therefore culture supernatant can be used for the synthesis of silver nanoparticles rather than cells itself (Gurunathan et al.,2009). Not all organisms have the ability for the synthesis of silver nanoparticles. The exact reaction mechanism leading to the formation of silver nanoparticles by all organisms is yet to be un known .The organisms which have the "Silver resistance machinery" can synthesize silver nanoparticles . Extracts from microorganisms may act both as reducing and capping agents in AgNPs synthesis (Kalimuthu, et

al.,2008). For example, Bhainsa and D'Souza (2006) studied extracellular synthesis of silver nanoparticles by A.fumigatus some study showed that nicotinamide adenine dinucleotide (NADH-) and NADH-dependent enzymes are important factors used in the biosynthesis of metal nanoparticles. The reduction appears to be initiated by electron transfer from the NADH by NADH-dependent reductase as electron carrier (Thakkar et al., 2010 ; Marambio et al.,2010) . Organisms which contain the "Silver resistance machinery" can synthesize silver nanoparticles provided that the concentration of the silver ions does not cross the "threshold limit". The resistance mechanism varies with organisms. Extracts from bio-organisms may act both as reducing and capping agents in AgNPs synthesis.

Characterization of AgNPs

Scanning Electron Microscopy

The results of SEM of nanosilver synthesized by E. faecium (Figur 2) showed different shapes and sizes of nanosilver , almost spherical in shape and some of these particles were in an aggregated form. The size of AgNPs ranges between 14.89 nm – 22.33 nm.Many studies proved the spherical shape of the synthesized nanosilver particles with size distribution ranging from 5 nm to 50 nm (Raza, et al., (2016) & Saifuddin et al., 2009). The previous study on the biosynthesize of silver nanoparticle from Lactobacillus sp. produce nanoparticle with size (30-100 nm) related with (Aldujaili et al.,2015 and Abdulhassan .,2016).



Figures 2: An electron micrograph of nanosilver synthesized by E. faecium at different magnification powers by Scanning electron microscope.

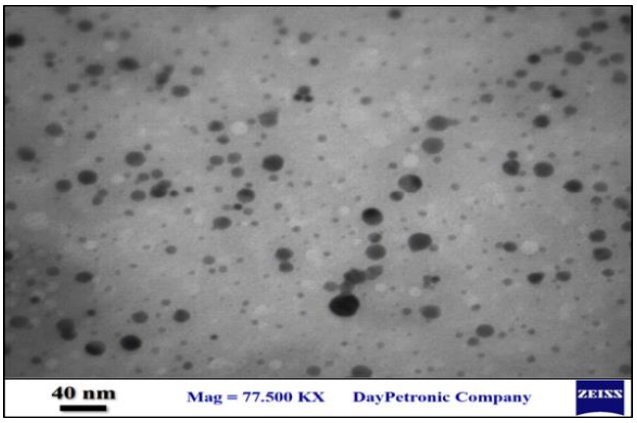


Figure 3: An electron micrograph of nanosilver synthesized by E. faecium at different magnification powers by Transmission electron microscope Fourier-Transform Infrared spectroscopy (FT-IR)

Transmission Electron Microscope (TEM)

The results of TEM (Figure 3) have demonstrated a polymorphic size and shape of AuNPs formed when mixing $AgNO_3$ solution with the supernatant of *E. faecium*. Also the results have shown the discrete nanosilver of less than 20 nm in diameter and they were mostly spherical indicating the possibility to synthesize silver particles of nano-dimension with a satisfactory level of mono–dispersity. This falls nearer to the range of silver nanoparticles, produced using *Enterococcus spp.*(Oladipo *et al.*,2017). The differences in the shape and size of nanosilver synthesized by *E. faecium* may be due to differences in the growth phase of particles (Rajeshkumar,2016; Dykman, and Khlebtsov, (2011).

Fourier transform infrared spectroscopy (FT-IR) measurements were used to determine if the biomolecules are susceptible to Ag+ ion reduction and identify the capping agent of the bioreduced AgNPs (Figure 4). The bands 3216 28.22 ,2092 10.19 and 1615 12.91 cm-1 correspond to the – NH2 of amines or -OH stretch of carboxylic acid, C=C stretch of alkenes, C=O stretch of amides or N-H band of 1° amine respectively. This is an indication that proteinous molecules present in the cell-free extracts of strain of Enterococcus were involved in the biosynthesis of AgNPs. (Rajeshkumar.2016;Oladipo et al.,2017;Sharma et al.,2012). Many researchers have reported that some functional groups, such as – NH2, -OH, -SH and -COOH, of the proteins secreted by bacteria, play important roles in the reduction and stabilization of NPs. These functional groups provide binding sites for fixing of metal ions, followed by the reduction the metal ions outside the cells on the cell wall or in the periplasmic space. In this study protein functional groups and other nitrogenous molecules such as -NH2, -OH, -SH and -COOH act as reduction and stabilization agents of Ag+ to Ag-NPs.

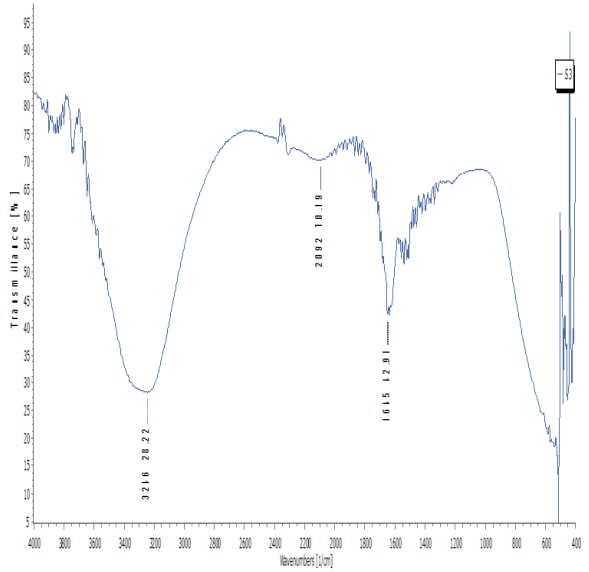


Figure (4) FT-IR chart of nanosilver particles synthesized by E.faecium (sample 2)

Dynamic Light Scattering (DLS)

The results of the DLS technique for the characterization of the sample of nanosilver synthesized by E. faecium have shown that the average of hydrodynamic particles size was 83.6 nm (Figure 5). Nanosilver showed a relatively uniform and monodisperse size distribution . However, the distribution curve split into two peaks ,one peak at large size around 100 nm, and another peak around 8 nm. The higher peak around a size of 100 nm in diameter may be due to formation of a high variety of similarly sized aggregates (Gunnarsson et al.,2019) .The large size particles observed by DLS was due to the bio-organic compounds enveloping the core of the silver nanoparticles confirmed by the FTIR results (Prathna et al.,2011). Gunnarsson et al., (2019) confirmed the difference between the hydrodynamic diameter and the absolute diameter by measuring the gold nanoparticle hydrodynamic size which was 108 nm and nacked gold nanoparticle was 78 nm ,indicating the biomolecules proteins of 15 nm. This increased diameter of the NP, and therefore increased scattering intensity gave a broad peak. This is an important indicator of in vitro assays, i.e. how rapidly it reaches the cells (Leroux et al.,1995; Oh and Park,2014).

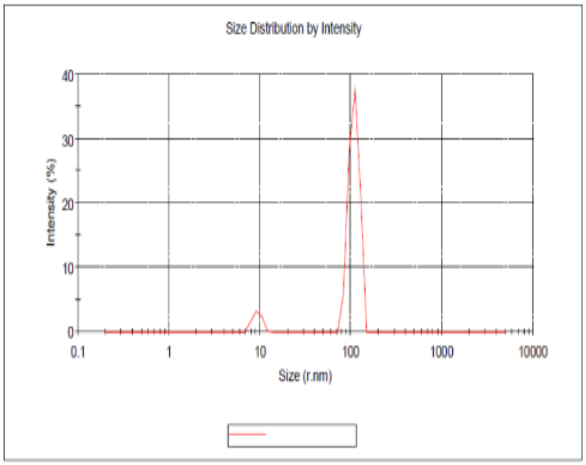


Figure (5): Dynamic Light Scattering(DLS) chart of nanosilver particles synthesis by E.faecium

X-Ray Diffraction (XRD)

XRD analysis was carried out using Nanosilver synthesized by E. faecium to determine the crystalline nature of Nanosilver. (Figure 6). The exact nature of the AgNPs can be deduced from the XRD spectrum of the sample. The XRD pattern showed four peaks at 2θ values of 38.1°, 44.3°, 64.4°, and 77.2°, corresponding to (111), (200), (220), and (311) respectively, in the whole spectrum of 2θ values ranging from 20° to 80°. A comparison of our XRD spectrum with the Standard (JCPDS file no 04-0783) confirmed the formation of crystalline AgNPs.The XRD results have shown presence of the Bragg peaks of elemental silver at 2Oθ, values in the bacterial supernatant powders . Other peaks seen in the spectra belong to the impurities present in the bacterial supernatant (Pourali et al.,2017). The presence of organic materials on the surface of the nanoparticles proves the presence of encapsulation from the core of the biomolecules present in the bacterial extract. This is consistent with the results of DLS due to the frequency of the nanoscale that this measurement proves that the repeatability of the measurement is around 100 nm.

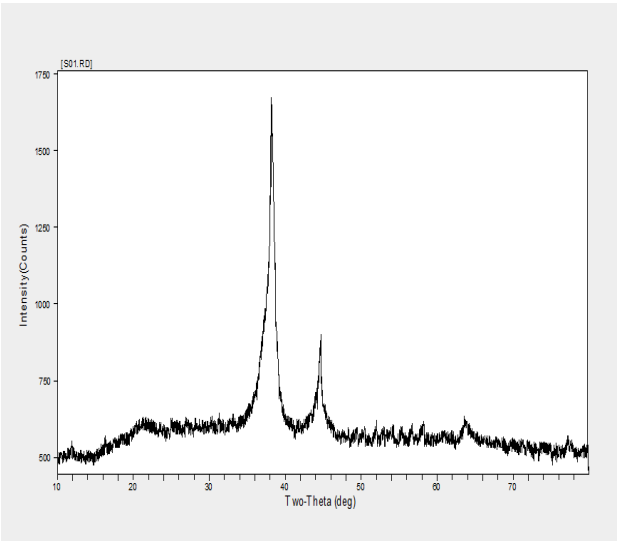


Figure (6): X-Ray Diffraction (XRD) chart of nanosilver particles synthesized by E.faecium

Antibacterial Activity of Nanosilver Synthesized by E.faecium

The results shown in Figure (7)and Table (1) revealed that the nanosilver were more influenced on K. pneumoniae at ahigh significant ($p \leq 0.05$) Compared to other bacterial isolates used in this study, as the main inhibition zone for K. pneumoniae was 29 mm ,while the other (20,19.18)mm for E. coli , Strep. mutans and Staph. epidermidis respectively . It appears that the obvious effect to on gram negative bacteria is more than gram positive ,which may be evidence that nanosilver have ability to penetrate G-ve bacterial cell wall more efficiency than G+ve bacteria , perhaps because of the ease of association with G-ve cytoplasmic outer membrane , while the entry of nanosilver into G+ve is disabled by presence of thick layer peptidoglycan . In the current study, green synthesis of AgNPs using the cell-free extracts of Enterococcus species was successfully carried out. The spherical biosynthesized AgNPs of 4-55 nm in size displayed good antibacterial activities against multi-drug resistant bacterial isolates . Because of the emergence and increase in the number of multiple antibiotic-resistant microorganisms, nanoparticles are now considered alternate to antibiotics and have a great perspective to resolve this problematic. AgNPs were mostly good-looking for the making of a novel class of antimicrobials (Pinto et al., 2009 & Rai, et al.,2012).

Klebsiella pneumoniae

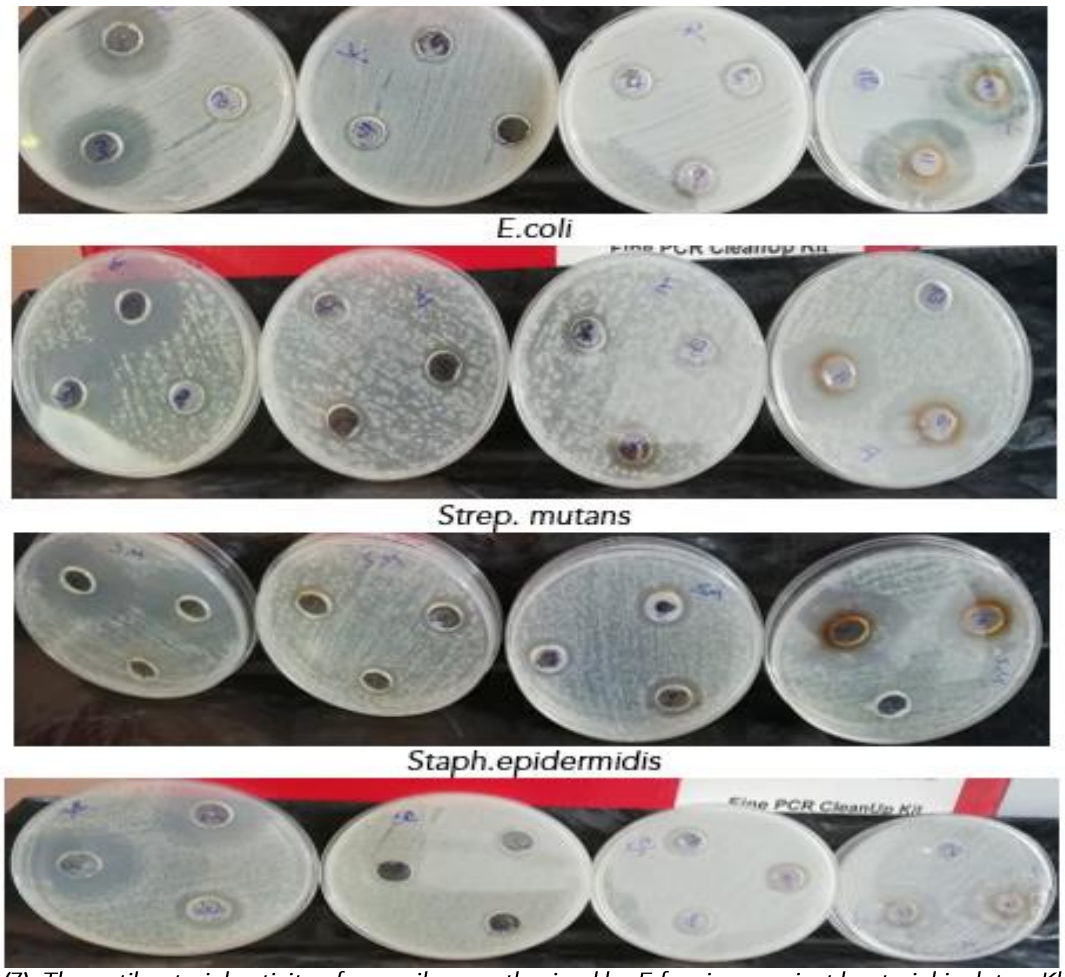


Figure (7) :The antibacterial activity of nanosilver synthesized by E.faecium against bacterial isolates, Klebsiella pneumoniae , E.coli , Strep. mutans , Staph. Epidermidis

Table (1) : Mean of inhibition zones against experimented bacteria	
Bacterial isolates	Inhibition zones(mm) Mean ± Sd
Klebsiella Pneumoniae	29 ± 9.4 *
E. coli	20 ± 6.2
Strep. Mutans	19 ± 4.25
Staph. epidermidis	18 ± 5.3

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