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RESEARCH ARTICLE

Antibacterial activity of silver nanoparticles Synthesized by *Lactobacillus* spp.against Methicillin Resistant-*Staphylococcus aureus*

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Methicillin -Resistant *Staphylococcus aureus* (MRSA) represent a major problem in human medicine by causing both healthcare-associated and community-associated infections .The present study focuses on the rapid synthesis of silver nanoparticles by Iraqi *Lactobacillus* spp. isolates (*Lactobacillus acidophilus* ,*L.gasseri*, *L.rhamnosus* , *L.fermentum*). The silver nanoparticles were characterized with Atomic Force Microscopy (AFM) and found the average size ranges from 33.71 to39.16 nm. Antibacterial activity of the silver nanoparticles were investigated against methicillin- resistant *Staphylococcus aureus*(MRSA) by using agar well diffusion method and Co-culture technique to calculated percent reduction of bacteria..Large zone of inhibitonseems and% reduction of MRSA growth are found to be 100% . To the best of our study this is the first report on synthesis of silver nanoparticles by Iraqi *Lactobacillus* isolates and detection of antibacterial activity against MRSA .

Introduction

The high prevalence of nosocomial infections due to multi resistant bacteria, such as found with methicillin-resistant *Staphylococcus aureus* (MRSA), is currently explained by intensive use of topical and systemic antimicrobial agents in health care settings, which represents a highly selective pressure for antibiotic-resistant bacterial clones. In addition, bacterial exposure to some antibiotic classes may potentially induce endogenous, resistance-conferring mutations in bacterial genes that encode drug targets(Didier *et al*.,2011).

Silver nanoparticles are among the most commercialized inorganic nanoparticles due to their antimicrobial potential. They have also been used for a number of applications such as non linear optics, spectrally selective coating for solar energy absorption, biolabelling and antibacterial activities(Ranganath*et al.*,2012).Production of silver nanoparticles can be achieved through different method, Chemical approaches are the most popular methods for the production. However, some chemical methods cannot avoid the use of toxic chemicals in the synthesis protocol. There is a growing need to develop environmentally friendly processes of nanoparticles synthesis that do not use toxic chemicals(Duran *et al*.,2005).Bio-synthesized silver nanoparticles displayed good antimicrobial efficacy towards Gram-negative and Gram-positive bacteria as well as anti-biofilm potency against biofilm pathogen(Mohsen *et al*.,2011; Kumar *et al*.,2012)

This is now well known that many organisms, can produce inorganic materials either intra- or extracellularly , Bacteria, being prokaryotes have survived the test of time in enriching ions , synthesizing magnetite nanoparticles, reducing Ag into metal particles, forming nanoparticles , and in generation of cermets(Prasad *et al*.,2007).The formation of extracellular and intracellular silver nanoparticles by bacteria (*Pseudomonas stulzeri*, *Escherichia coli*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Salmonella typhus*, and *Staphylococcus currens*) has beeninvestigated(Lengke*et al*.,2007). Nanocrystals of gold, silver and their alloys have been synthesizedby the assistance of lactic acid bacterial cells (Prasad *et al*.,2007).

In this paper, we report on the synthesis of silver nanoparticles by some locally *Lactobacillus* spp. and detection of its antibacterial activity against MRSA.

Materials and Methods

-*Lactobacillus spp.* Isolates:

Lactobacillus isolates used in the study included (*Lactobacillus acidophilus ,L.gasseri, L.rhamnosus* and *L.fermentum*) isolated from Vaginal and Stool samples obtained from Department of biology/College of Science/Al-Mustansiriya University/Baghdad / Iraq.

- Methicillin- resistant *Staphylococcus aureus*(MRSA)

Isolate of MRSA was identified by conventional biochemical reactions and Gram staining, according to the criteria established by (Forbes *et al* ., 2002). Antimicrobial susceptibility of the isolate was tested by the disc diffusion method for Methicillin(10µg) (Bioanalyse, Turkey) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI,2009) .

Synthesisof silver nanoparticles by*Lactobacillus* spp.:

In a typical procedure of nanoparticles synthesis, every isolate were individually inoculated into sterilized 250 ml of whole milk in 500 ml flask and incubated for curdling at 37°C for 24 hours. The whey was collected by coarse filtration (Whatman 40). The filtrate was pale yellow in appearance, and the pH was typically 4.4. To 5 mL of each sample solution taken in a test tube, 1 mg of AgNO3 was added and kept in the laboratory under ambient conditions . The solution became brown in about 12-16 h(Ranganath*et al.*,2012). A brown mass gets at the bottom of the test tube after 24,48,72 h depending on isolates. Control was whey without AgNO3.

Characterization of silver nanoparticles by Atomic Force Microscopy:

Atomic Force Microscopy image was taken using Park system AFM XE 100. The aqueous silver nanoparticles were deposited onto a freshly cleaved mica substrate. The sample aliquot was left for 1 min and then washed with deionized water and left to dry for15 min. The images were obtained by scanning the mica in air innon – contact mode (Daniel *et al.*,2012).

Antibacterial activity of silver nanoparticles produced by *Lactobacillus* isolates:

-Agar Well diffusion- method:

Silver nanoparticles produced by *Lactobacillus acidophilus, L.gasseri, L.rhamnosus ,L.fermentum*were screened for their inhibitory activities against MRSA, using agar well diffusion-method. Plates were prepared by spreading approximately 10^5cfu/mlculture broth of indicator bacterial isolate on nutrient agar surface. The agar plates wereleft for about 15 min before aseptically dispensing the 50µl of each silver synthesized by *Lactobacillus* isolates into the agar wells already bored in the agar plates. The plates were then incubated at 37°C for 18 - 24 h. Zones of inhibition were measured and recorded in millimeter diameter.

Co-culture technique

Co-culture assay,another method for determination of antibacterial effect of Silver nanoparticles synthesized by *Lactobacillus acidophilus ,L.gasseri, L.rhamnosus , L.fermentum*separately.The bacterial culture of MRSA was grown in nutrient broth with different ratio of silver nanoparticles synthesized by *Lactobacillus* isolates separately (2:2,1.5:2.5,1:3) (silver :nutrient broth), the control medium contained nutrient broth only. Co-cultures and control were incubated at 37^oC for 24 h. After the incubation 1ml of each cultures were serially diluted upto 10^{-1} to 10^{-7}. Then 0.1ml of each dilution sample was taken and spreaded on nutrient agar plates. The plates were incubated at37^oC for (24 – 48) h. The colonieswere counted and the inhibition activitywas evaluated and calculated percent reduction of bacteria using the following equation described as Gosh *et al.*,(2010) :

R(%)=[A−B]/A ×100

R=the reduction rate,A= the number of bacterial colonies from control medium and B= the number of bacterial colonies from treated with silver nanoparticles

Results and Discussion

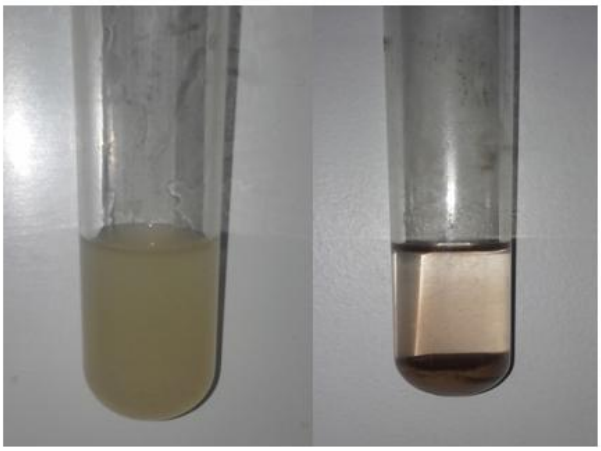
Synthesis and Characterization of silver nanoparticles

In the present study all *Lactobacillus spp* isolates (*L.acidophilus , L.gasseri , L.fermentum* and *L.rhamnosus*), confirmed as positive for Biosynthesized silver nanoparticles by change of the reaction mixture from pale yellow to brown color (Fig 1) indicating the production of silver nanoparticles (Ag+ to Ag0) this result agree with Minaeian*et al*.,(2008).

A brown mass gets at the bottom of the test tube after 24h for *L.gasseri* and 48h for *L.fermentum* while the isolates *L.acidophilus* and *L.rhamnosus* produced a brown mass after 72 h.

It is reported that reduction of Ag+ to Ag0 occurs through nitrate reductaseenzyme , these enzymes released in the solution can reduce the silver nitrate to silver nanoparticles through capping agents such as proteins (Ranganath*et al.*,2012).Lactic acid bacteria including *Lactobacillus* spp.,

Pediococcuspentosaceus, *Enterococcus faecium*, and *Lactococcusgarvieae*, were able to reduce silver, and *Lactobacillus* spp. can be used for a rapid and efficient production of silver nanoparticles (Sintubin *et al*.,2009).Biosorption and bioreduction of Ag° on cell surface was also reported in *Lactobacillus* spp. at 30° C, pH 4.5 in 24 h by Lin *et al.*,(2005).



a= Controlb= test

Fig 1: Lactobacillus spp in whey (a) without AgNO3 taken as control (b) with AgNO3 (1mg/5 ml).

The knowledge about the reduction of silver ions and formation of silver nanoparticles were still not clear, but believe that protein molecules and enzyme, includes nitrate reductase enzyme act as good regulating agent in silver nanoparticles synthesis.The primary conformation of synthesis of nanoparticles in the medium was characterized by the changes in color from yellowish white to brown(Natarajan*et al*.,2010).

Silver nanoparticles of well defined morphology and size are formed within the periplasmic space of the bacteria. The biogenic method for nanoparticle production is simple, eco-friendly and allows for getting controlled nanoparticles which can be used as catalysts with specific composition, which cannot be synthesized by classical methods. Applications in sensors and medicine are envisaged and the nanoparticles synthesized in the bacteria can be used against the human pathogens (Popecu*et al*.,2010).

This study coveredparticle size was analysed by Atomic Force Microscopy. AFM was used to view the nanoparticles both in surface and three Dimensional view ,and found theaverage size of particles(37.12, 34.62,39.16,33.71) nm. synthesizedby*L.acidophilus*,*L.fermentumL.gasseri*,*L. rhamnosus*) respectively Fig. (2,3,4,5).

Korbekandi*et al*.,(2012) showed that the biosynthesized silver nanoparticles were almost spherical, single (25–50 nm) or in aggregates (100 nm), attached to the surface of biomass or were inside and outside of the cells.

Antibacterial activity of silver nanoparticles against MRSA:

Antibacterial activity of the silver nanoparticles synthesized by *Lactobacillus spp* isolates were tested against MRSA using well diffusion technique. The diameter of inhibition zones around each well with silver nanoparticles is represented in (Fig.6). The highest antibacterial activity was observed by silver of *L.gasseri* follow*by* silver of *L.rhamnosus* .Moreover, all the silver nanoparticles synthesized by *Lactobacillus spp* isolates at different ratio (2:2,1.5:2.5,1:3) showed high inhibition activity and no colonies of MRSA observed from silver nanoparticles treated samples found to (B)= zero, so bacterial colonies from control(untreated) found to (A)=38. % reduction of MRSA growth are found to be 100%.These results suggest that silver nanoparticles synthesized by *Lactobacillus* spp. could be used as an effective antibacterial material against MRSA. MRSA infections cause a number of diseases, including osteomyelitis, bacteraemia, chronic wound infection,septic arthritis, skin and soft tissue infections, chronic rhinosinusitis (CRS), and pneumonia. Such staphylococcal infections are becomingly increasingly difficult to treat(Leid*et al.*,2012). In particular, silver ions have long been known to exert strong inhibitory and bactericidal effects as well as to possess a broad spectrum of antimicrobial activities(Soo-Hwan *et al*.,2011).

Shahverdi*et al*.,(2007); Shirley*et al.*,(2010) ;Mohsen*et al* .,(2011) ; Soo-Hwan *et al*.,(2011) showed that the silver nanoparticleshave potent antibacterial activities against*S. aureus and E. coli*. Nada and Saravanan (2009) showed that silver bionanoparticles from bacteria have inhibitory and bactericidal effect against MRSA. The mechanism of inhibitory action of silver ions on microorganismsispartially known. It is believed that DNA loses its replication ability and cellular proteins become inactivated on Ag+ treatment. In addition, it was also shown that Ag+ binds to functional groups of proteins, resulting in protein denaturation(Gash *et al.*,2010).

The silver atoms bind to thiol groups (-SH) in enzymes and subsequently cause the deactivation of enzymes. Silver forms stable S-Ag bonds with thiol-containing compounds in the cell membrane that are involved in transmembrane energy generation and ion transport (Kluhe*et al* .,2000). It is also believed that silver can take part in catalytic oxidation reactions that result in the formation of disulfide bonds (R-S-S-R). Silver does this by catalyzing the reaction between oxygen molecules in the cell and

hydrogen atoms of thiol groups: water is released as a product and two thiol groups become covalently bonded to one another through a disulfide bond (Davies *et al*.,1997).

Another one of the suggested mechanisms of the antimicrobial activity of silver was proposed that Ag^{+} enters the cell and intercalates between the purine and pyrimidine base pairs disrupting the hydrogen bonding between the two anti-parallel strands and denaturing the DNA molecule (Ranganath*et al.*,2012).

Fig. (1). Atomic Force Microscopy image of silver nanoparticles synthesizedby *L.acidophilus*.

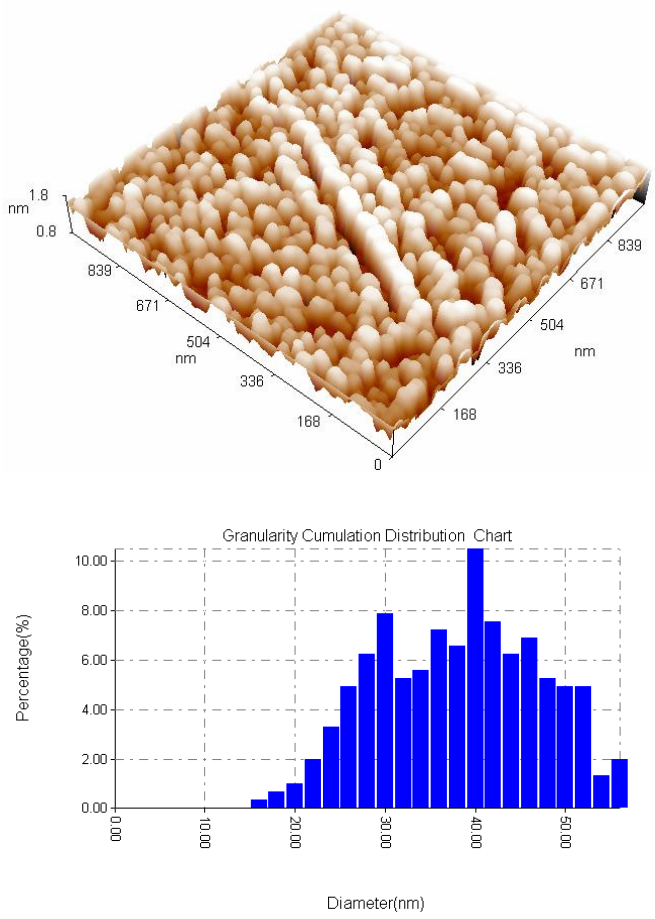


Fig. (2). Atomic Force Microscopy image of silver nanoparticles synthesizedby*L.fermentum*.

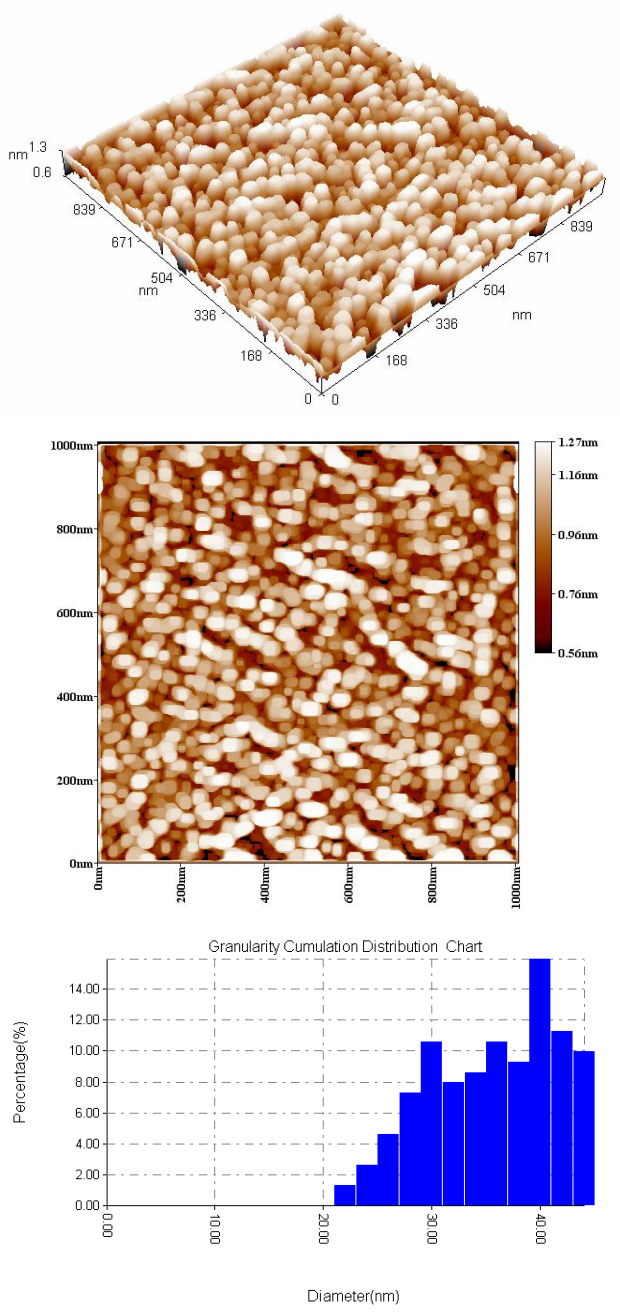


Fig. (3). Atomic Force Microscopy image of silver nanoparticles synthesized by *L. gasseri*.

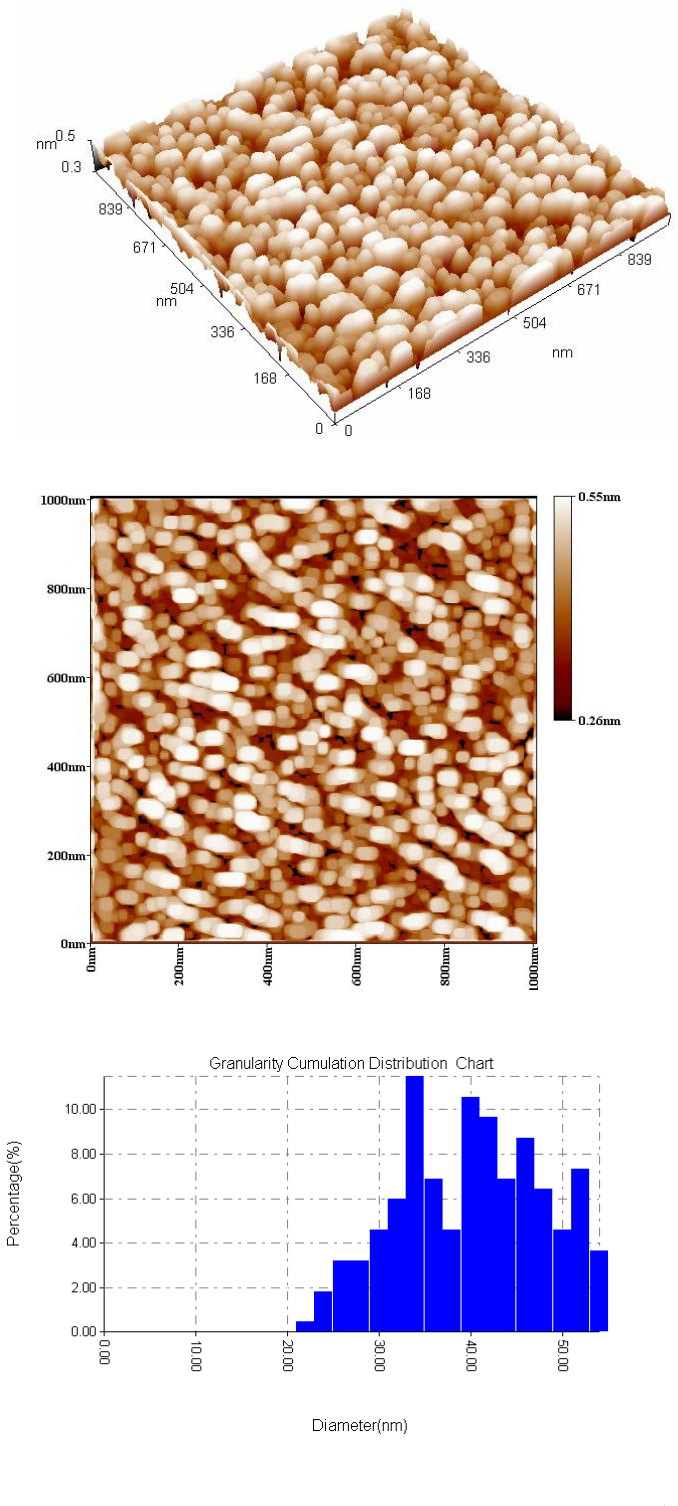


Fig. (4). Atomic Force Microscopy image of silver nanoparticles synthesized by L. rhamnosus

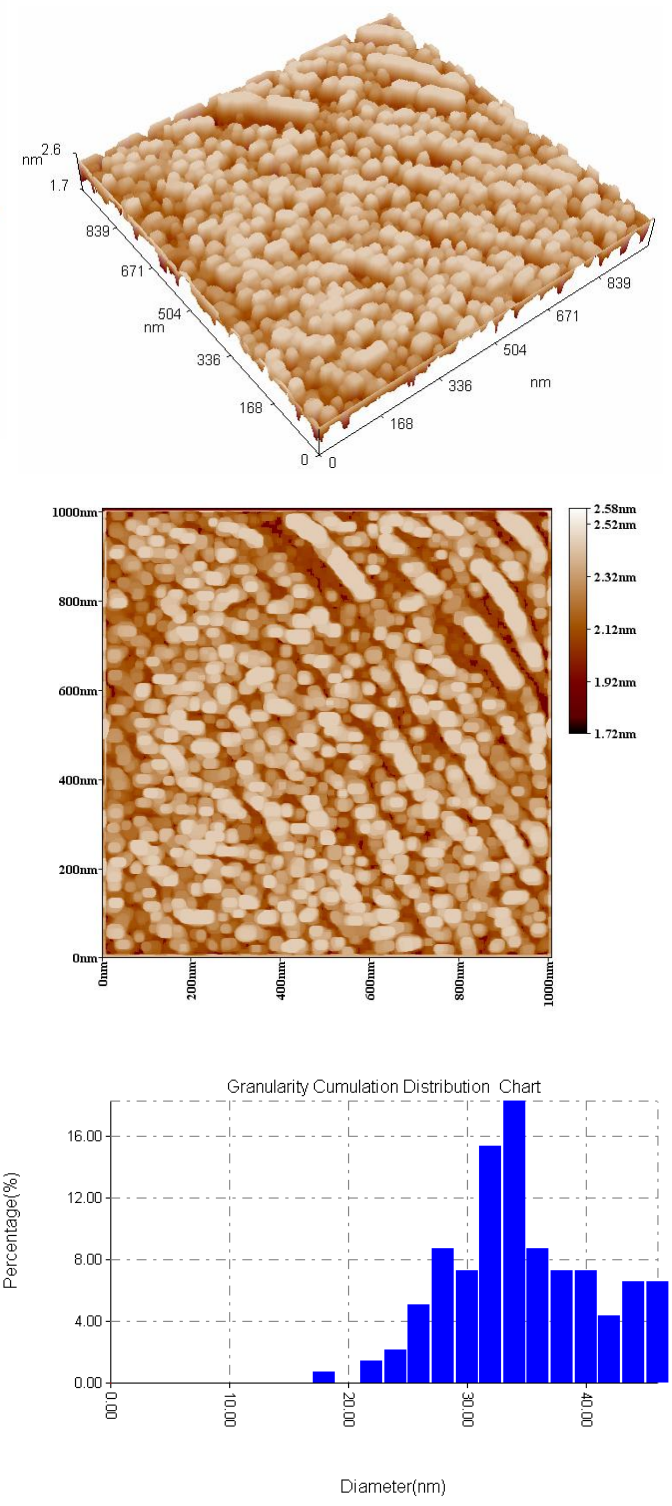
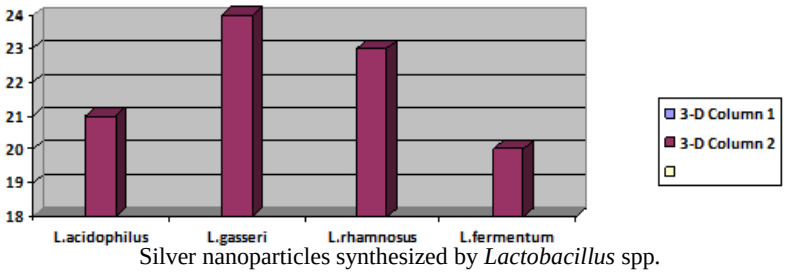


Fig. (5):Inhibition zone of of silver nanoparticles synthesized by *Lactobacillus* spp. against MRSA



Silver nanoparticles synthesized by *Lactobacillus* spp.

Conclusion:

The present study demonstrated the synthesis of silver nanoparticles using locally *Lactobacillus*isolates (*L.acidophilus* ,*L.gasseri* , *L.fermentum* and *L.rhamnosus*). The synthesized silver nanoparticles were characterized by using Atomic Force Microscopy. Antibacterial activity was observed against MRSA.

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