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CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF SILVER NANOPARTICLES SYNTHESIZED BY RICE STRAW UTILIZING BACTERIUM (*LYSINIBACILLUS FUSIFORMIS)*

***Naeima M. H. Yousef**

Botany and Microbiology Department, Faculty of Science, Assiut University, Assiut, Egypt

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ABSTRACT

Use of microorganisms in synthesis of silver nanoparticles (AgNPs) is coast effective and eco-friendly and easily scaled up for large scale synthesis. The present investigation reports a simple and environmental friendly method for biological synthesis of silver nanoparticles (AgNps) by *Lysinibacillus fusiformis* strain AUMC b-160 which was isolated from soil sample treated with manure and showed ability to utilize wheat and rice straws as a sole carbon source. The bacterium has been identified molecularly by partial sequencing of the 16s rDNA gene (approximately 1100 bp) and the result demonstrate 99% homology to *Lysinibacillus fusiformis.* Furthermore, the nucleotide sequence deposited in the GenBank under accession number KJ571527. The biosynthesized nanoparticles are characterized by the UV-Vis spectroscopy that revealed the characteristic surface plasmon resonance band centered at 425 nm. Transmission electron microscopy (TEM) analysis confirmed the formation of varying morphology silver particles in the size range from 10-22 nm. FTIR confirmed that the synthesized nanoparticles are surrounded by proteins and metabolites and the stability of silver nanoparticles are due to proteins and enzymes coated them. Also the study was designed to compare the in *vitro* antimicrobial effect of biological silver nanoparticles against human pathogenic bacteria and *Candida*. It was found that the growth of all using bacteria and *Candida* was inhibited by the biosynthesized silver nanoparticles.

INTRODUCTION

The development of biological experimental processes for the synthesis of nanoparticles is evolving into an important branch of nanotechnology (Ahmed *et al*., 2003). Biologically synthesized silver nanoparticles have many applications such as antimicrobial agents (Jeevan *et al*., 2o12). The metallic nanoparticles are most promising as they have antibacterial activity due to their large surface area to volume ratio, which is of great interest to researchers due to the growing microbial resistance against metal ions, antibiotics, and the development of resistant strains (Rai *et al*., 2009). The advantage of AgNPs over conventional antibiotics in that it has ability to kill all pathogenic microorganisms, and no microorganism has ever been reported to readily develop resistance to it (Dameron *et al*., 1989). Silver nanoparticles are used as antimicrobial agents in surgically implanted catheters in order to reduce the infections caused during surgery and are proposed to possess antifungal, antiinflammatory, antiangiogenic and anti permeability activities (Gurunathan *et al*., 2009). Primarly silver nanoparticles are considered as an alternative to silver ions (obtained from silver nitrate), which were used as antimicrobial agents (Morones *et al*., 2005). Among the many microorganisms, bacteria have received the most publicity in the area of biosynthesis of metal nanoparticles. Klaus *et al*. (1999) and Joerger *et al*. (2000) have shown that *Pseudomonas stutzeri* AG259, which was isolated from a silver mine, has ability to reduce aqueous silver nitrate solutions to nanoparticles with the diameter of 3–200 nm. Shahverdi *et al*. (2007) demonstrated the rapid formation of silver nanoparticles by the culture supernatants of different Enterobacterial strains and suggested that the nitroreductase enzymes may be involved in the reduction process of silver ions. Kalimuthu *et al*. (2008) found also that *Bacillus licheniformis* can biosynthesize of silver nanoparticles with average particle size 50 nm and concluded that the enzyme involved in the bioreduction of Ag+ ions and the formation of silver nanoparticles may be nitrate reductase because *B. licheniformis* is known to secrete the cofactor NADH and NADH dependent enzymes, especially nitrate reductase. Many

****Corresponding author: Naeima M. H. Yousef***
Botany and Microbiology Department, Faculty of Science, Assiut University, Assiut, Egypt

studies using bacterial culture supernatants like *Bacillus megaterium*; *Pseudomonas proteolytica*; *Pseudomonas meridian*; *Pseudomonas aeruginosa; Arthrobacter kerguelensis*; *Bacillus indicus: Sphingobacterium muzetti* were proven to form extracellular nanoparticles very effectively (Saravanan *et al*., 2011; Shivaji *et al*., 2011; Jeevan *et al*., 2012; Yousef and Nafady 2014). This study focused on the characterization of biological synthesis of silver nanoparticles using culture supernatant of *Lysinibacillus fusiformis* which showed ability to utilize wheat and rice straws as a sole carbon source. The study was also designed to know the evaluate their antimicrobial effect in *vitro* against some human pathogenic bacteria and *Candida*.

MATERIALS AND METHODS

Isolation and molecular identification of the bacterium strain

A bacterial strain was isolated from an Egyptian soil sample fertilized with manure. The soil sample was suspended in nutrient broth throughout serial dilution then plated onto nutrient agar for 24 h. The colonies were counted, then picked out and purified by streaking on nutrient agar plates. Pure cultures were tested for their ability to utilize wheat or rice straws as a sole carbon source using minimal medium supplemented with 10g|L wheat or rice straws.

Molecular identification

DNA extraction, purification and amplification

DNA of using bacterium was extracted and purified as described previously by Mohamed *et al*. (2012). Polymerase chain reaction (PCR) was performed using a thermal cycler (Tpersonal-Biometra-Germany) in the Molecular Biology Research Unit, Assiut University. PCR was carried out using universal primers F8 and R1525 according to Beumer and Robinson (2005). Primers were manufactured by eurofins MWG operon, Ebersberg, Germany. The PCR reaction was performed in a total volume of 50 µl by using 20 µl 2.5X PCR Master mix (Prime 5), 3 µl from forward and reverse primer (10 pmol/µl), 23 µl nuclease free water and 1 µl (100 ng) DNA. A negative control was performed as the same but without template DNA. Initial denaturation was at 94ºC for 4 min, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 51ºC for 1 min and extension at 72ºC for 90 sec, with a final extension at 72ºC for 7 min. For Gel electrophoresis, five ml of the amplified PCR product was loaded using 1% agarose gel electrophoresis as reported by Sambrook *et al*. (1989). After adequate migration has occurred, DNA fragments were visualized on an ultraviolet transilluminator (Biometra, Germany).

DNA sequencing

DNA product was purified using Qiagen DNA purification Kit and sequenced in both directions using 27F and 1492R primers in an ABI 3730 automated sequencer. The bacterial 16S rDNA sequences obtained were then aligned with known 16S rDNA sequences in Genbank database using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/), and percent homology scores were generated to identify bacteria.

Data analysis and phylogenetics

A phylogenetic tree was constructed with MEGA version 4.0 using a neighbor-joining algorithm, plus the Jukes-Cantor distance estimation method with bootstrap analyses for 1,000 replicates was performed.

Nucleotide sequence accession number

The sequencing of the gene coding for 16S rRNA gene for the strain has been deposited in the GenBank nucleotide sequence databases (NCBI) under Accession number and the strain was also deposited in the culture collection of Assiut University Mycological Centre, Assiut, Egypt as AUMC b-160.

Biological synthesis of silver nanoparticles

The silver nanoparticles were synthesized as described previously by Yousef and Nafady (2014). *Lysinibacillus fusiformis* was grown in nutrient broth (peptone 10 g/L, yeast extract 5 g/L and NaCl 5 g/L) and incubated in an orbital shaker (80 rpm) for 24 hours at 30°C. Then, the culture was centrifuged at 8000 rpm for 15 min and the cell-free supernatant was used for the biosynthesis of AgNPs. Aqueous silver nitrate solution (1 mM) was mixed carefully with 50 mL of cell supernatant in 250 mL conical flask and agitated in shaker (80 rpm) at room temperature. Control without the $AgNO_3$ (cell-free supernatant) was also kept at the same conditions.

Characterization of silver nanoparticles

UV-visible spectroscopy analysis

To confirm the formation of silver nanoparticles, the optical properties of silver nanoparticles were carried out on Ultraviolet-Visible Spectroscopy (UV-Vis) (Evolution 300-UV-Visible spectrophotometer- England) at a resolution of 1 nm and scanning the spectra between 300-900 nm. A strong absorption of electromagnetic waves is exhibited by metal nanoparticles in the visible range due to the surface plasmon resonance. The stability of stored biologically synthesized silver nanoparticles was also performed by UV-Visible spectral analysis.

Transmission Electron Microscope (TEM)

The morphology and sizes of silver nanoparticles was determined by TEM micrographs using the JEOL TEM 100 CXII (Electron Microscope Unit, Assiut University, Egypt). The sample was prepared by placing a drop of synthesized silver nanoparticles on a negative carbon coated copper grids and dried in air.

Fourier transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy measurements were carried out to determine the interaction between protein and metallic particle from the absorption of IR radiation through resonance of non-

centro symmetric modes of vibration and confirm the presence of protein for reduction, capping and efficient stabilization of the silver nanoparticles synthesized by *Lysinibacillus* culture supernatant. To investigate the functional groups possible binding sites with nanoparticles using FTIR spectroscopy, the solution of AgNPs was centrifuged at 9000 rpm for 20 min. The pellet was washed with ethanol for three times and then air-dried to obtain dried powder. FTIR spectra was obtained by KBr pellets methods operated on FTIR Infrared spectrophotometer (IR 470- Shimadzu- Japan). FT-IR spectra over the scanning range of 4000-400 cm^{-1} were obtained with the resolution of 2 cm^{-1}.

Antimicrobial activity of the synthesized silver nanoparticles

After characterization of silver nanoparticles, their antimicrobial effect was checked by disc diffusion method (Boyce 1984) on some common pathogens of human. The test microorganisms were *Escherichia coli* ATCC 8739, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* BM14, *Enterococcus sp.* BM15, *Klebsiella pneumoniae* BM12, *Serratia marcescens* W225 and *Candida albicans* AUMC 8175. Nutrient agar and Sabouraud agar media were used for growing the test bacteria and *Candida*, respectively. The antimicrobial spectrum of the synthesized silver nanoparticles was determined by the disc diffusion method on plates seeded with the tested microorganisms. Overnight cultures of the bacterial and *Candida* were prepared in nutrient broth or sabouraud broth and diluted to $1x10^5$ CFU|ml, 200 µl were stirred well in nutrient or sabouraud agar plates. After solidification, three sterile paper disks were placed on the surface of each agar plate and were impregnated with 10 µl of the silver nanoparticles or bacterial supernatant or 1mM silver nitrate. The plates were then incubated at 30ºC for 24 hours, then the diameters of inhibition zones were measured to estimate their inhibitory effects. The the mean values of inhibition zones were recorded.

RESULTS AND DISCUSSION

The use of microorganisms in the synthesis of nanoparticles is a relatively new and exciting field of nanotechnology. In most cases, microorganisms were identified through the addition of metal salts to the growth media or the cell extract followed by monitoring cells or the medium for the presence of nanoparticles. The bacterial strain *Lysinibacillus fusiformis* AUMC b-160 was isolated from soil sample among several bacterial colonies and showed ability to utilize wheat and rice straws as a sole carbon source as well as form silver nanoparticles as observed by change in the colour of the reaction within 10 min. The selected strain was subjected to molecular identification by partial sequencing of the 16s rDNA gene. The 16s rDNA was amplified (Fig. 1) and subjected to purification and sequencing. The sequence (approximately 1100 bp) demonstrate 99% homology to *Lysinibacillus fusiformis* (Fig. 2). Furthermore the nucleotide sequence was submitted to GenBank in NCBI with the accession number. Silver ions and silver salts are used as antimicrobial agents (Russel and Hugo 1994). However, the high concentration of silver salts restrict their uses. Use of metal nanoparticles decreases the concentration of silver and other metal salts. The antimicrobial effect of metal nanoparticles attributed to their small size and surface to volume ratio which allows them to interact closely with microbial membrane (Morones *et al*., 2005). There are several physical and chemical methods for synthesis of metallic nanoparticles (Edelstain & Cammarata 1996). However, development of simple and ecofriendly biological systems would help in the synthesis and application of metallic nanoparticles. Some microorganisms interact with metal ions reducing them into metallic nanoparticles (Prasad and Jha 2010). The biosynthesis of silver nanoparticles by the culture supernatants of *Lysinibacillus fusiformis* was carried out. The cell-free filtrate of the tested bacterium incubated with silver ion (1mg) is represented in Fig. 3. Rapid appearance of a yellowish –brown colour within 10 minutes in the reaction mixture suggested the formation of colloidal silver nanoprticles (shahverdi *et al*., 2007).

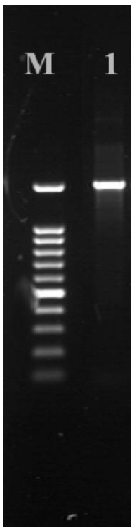

Fig. 1. Gel electrophoresis of the 16s rDNA gene. M, 100bp genetic marker, lane 1 is amplified PCR for *Lysinibacillus fusiformis*

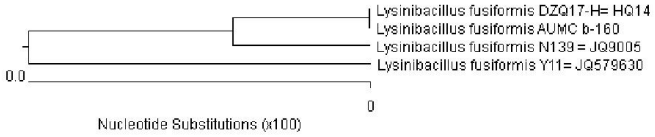

Fig. 2. The phylogenetic homology tree based on multiple sequence alignments of the 16s rDNA of the *Lysinibacillus fusiformis* reference to international isolates

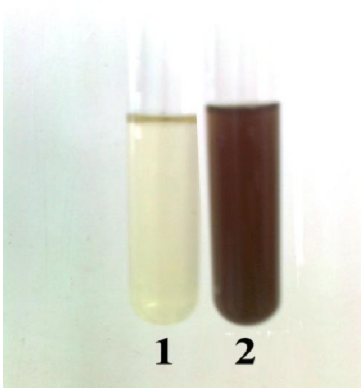

Fig. 3. Photograph of cell free bacterial supernatant before adding $AgNO_3$ (1) and after 24 hrs of adding $AgNO_3$ (2). Colour change indicates formation nanoparticles

Visual observation showed that the cell-free supernatant changed in intensity giving brown colour after 24 h of incubation. This increase in intensity could be due to the formation of more nanoparticles, that colour depends on the particle's size, shape and composition and the presence of adsorption layers and their structure (Krutyakov *et al*., 2008).
The UV-Visible absorption spectra of the silver nanoparticles in cell free supernatant were measured in the range 300-900 nm; Fig. 4 shows a strong broad absorption band located between 405-430 nm for silver nanopraticles prepared by the tested bacterium after 12 and 24 hours incubation. Increase in absorbance indicates that amount of silver nanoparticles increases. A typical nanoparticles absorption band in the visible region between 350 and 550 nm, Plasmon resonance peak obtained at 420 nm is well-documented for various metal nanoparticles with sizes from 2-100 nm (Sastry *et al*., 1998). The silver nanoparticles band remained around 420 nm indicating that the particles were well dispersed without aggregation.

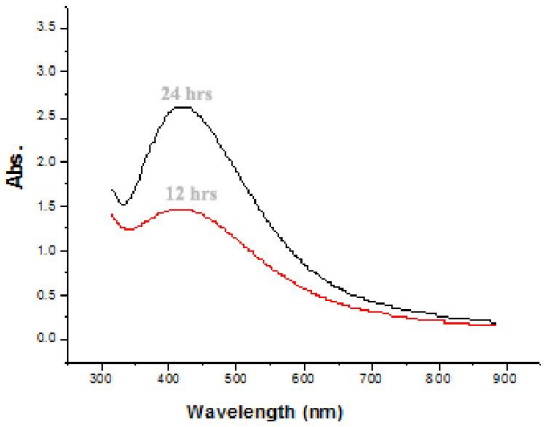

Fig. 4. UV-Vis spectrum of silver nanoparticles produced by *Lysinibacillus fusiformis* supernatant after incubation time 12 and 24 hours

Transmission Electron Microscope (TEM)

Fig. 5 shows the TEM images of silver nanoparticles synthesized using *Lysinibacillus fusiformis* culture supernatant. The particles are spherical shape with varying size AgNPs in the range from 10-22.6 nm, that can be used in several applications (Klaus *et al*. 1999).

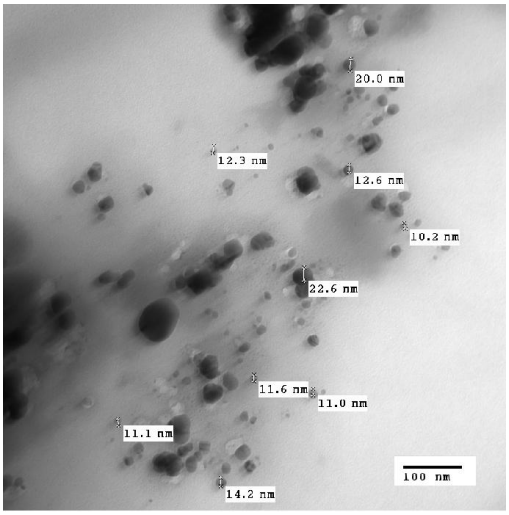

Fig. 5. TEM images of silver nanoparticles (AgNPs) synthesized using *Lysinibacillus fusiformis* culture supernatant

Fourier transform infrared spectroscopy (FTIR)

The FTIR confirmed that the carbonyl groups (C=O) from amino acid residues and peptides of proteins has the stronger ability to bind silver ions (Fig. 6). The FTIR spectra show the presence of two bands at 1650 and 1550 cm−1 which are identified as the amide I and II bands and arise due to carbonyl stretch and N H stretch vibrations in the amide linkage of the proteins, respectively (Ganesh Babu and Gunasekaran 2009).

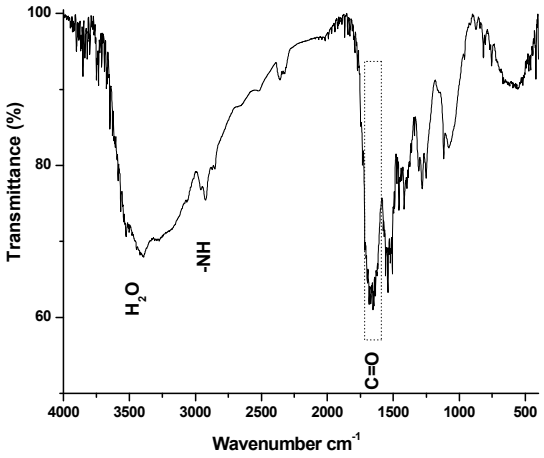

Fig. 6. FTIR spectrum of silver nanoparticles (AgNPs) synthesized using *Lysinibacillus fusiformis* culture supernatant

The previous studies reported that protein can bind to silver nanoparticles through their free amine groups or cysteine residues (Gole *et al*., 2001). The free amine and carbonyl groups present in the bacterial protein could possibly perform the function for the formation and stabilization of silver nanoparticles and prevent the aggregation of the particles (Balaji *et al*., 2009; Kasthuri *et al*., 2009). The amide linkage between amino acid residues in polypeptides represents a well-known signature in the infrared region of the electromagnetic spectrum. The FTIR Characterization of synthesized silver nanoparticles confirms the presence of proteins surrounding the nanoparticles. The antimicrobial activity of biosynthesized silver nanoparticles against pathogenic microorganisms (table 1) showed high effect against *E.coli*.

Table 1. Effect of synthesized silver nanoparticles on the inhibition growth of different microorganisms. Zone inhibition in mm, mean of triplicate

Microorganism	Biosynthesized silver nanoparticles (AgNPs)	Silver nitrate ($AgNO_3$) 1 mM
E. coli ATCC 8739	50	30
B. subtilis ATCC 6633	24	15
St. aureus BM14	45	35
K. pneumoniae BM12	23	21
Enterococcus sp. BM15	34	24
Serratia marcescens W225	32	28
Candida albicans AUMC	12	-

The silver nitrate showed also inhibitory effect on different human pathogenic bacteria and *Candida*. The inhibitory effect of silver nanoparticles is due to their action on the DNA and inactivation of celluar protein (Feng *et al*., 2000). In addition, it was suggested that silver ions bind to functional proteins, resulting in protein denaturation (Sondi and Matigevic 2003,

Sondi and Salopek-Sondi) and this was revealed when *E. coli* was treated with highly reactive metal oxide nanoparticles, a bacterial membrane exhibits a significant increase in permeability, leaving the bacterial cells incapable of properly regulating transport through plasma membrane and causing cell death. The mode of action of both silver nanoparticles and silver ions were reported to be similar although the nanoparticles were reported to be effective at siginficantly lower concentration than that of ions (Morones *et al*., 2005).

Conclusions

The biological synthesis of silver nanoparticles is cheap, eco-friendly and pollutant free. The culture supernatant of *L. fusiformis* plays an important role in reduction and stabilization of AgNPs. Further, the synthesized silver nanoparticles exhibit antimicrobial effect on Gram negative and Gram positive bacteria as well as *Candida albicans*. Silver nanoparticles exhibit completely new or improved properties compared to larger particles of bulk materials. Consequently, these particles have several important applications.

REFERENCES

Ahmad A, Mukherjee P, Senapati S, Mandal D, Khan MI, Kumar R, Sastry M 2003. Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium oxysporum*. *Coll Surf* B 28: 313–318.

Balaji D., Basavaraja S., Deshpande S., Bedre R., Mahesh D., Prabhakar B.K., Venkataraman A. 2009. Colloids Surf. B: Biointerfaces 68 88–92.

Beumer A., Robinson J.B. 2005. A broad-host-range, generalized transducing phage (SN-T) acquires 16s rRNA genes from different genera of bacteria. *Appl. Environ. Microbiol*. 71 (12) 8301-8304.

Boyce J M 1984. Reevaluation of the Ability of the Standardized Disk Diffusion Test to Detect methicillin resistant strains of *Staphylococcus aureus. J Clin Microbiol* 19: 813-817.

Dameron CT, Reese RN, Mehra RK, Kortan AR, Carroll PJ, Steigerwald ML, Brus LE, Winge DR 1989. Biosynthesis of cadmium sulfide quantum semiconductor crystallites. *Nature* 338:596–597.

Edelstein AS and Cammarata RC 1996. (Eds.), Nanomaterials: synthesis, properties and applications, IOP publication, Bristol and Philadelphia.

Feng QL, Wu J, Chen GQ, Cui FZ, Kim TN, Kim JO 2000. A Mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus. J Biomed Materials Res* 52: 662-668.

Ganesh Babu MM, Gunasekaran P 2009. Production and structural characterization of crystalline silver nanoparticles from *Bacillus cereus* isolate. *Coll. Surf*. B 74:191–195.

Gole A, Dash C, Ramakrishnan V, Sainkar SR, Mandale AB 2001. Pepsin-gold conjugates: preparation, characterization and enzymatic. Langmuir 17: 1674-1679.

Gurunathan S, Kalishwaralal K, Vaidyanathan R, Deepak V, Pandian SRK, Muniyandi JH, Hariharan N, Eom SH 2009. Biosynthesis, purification and characterization of silver nanoparticles using *Escherichia coli*. *Coll Surf* B 74:328–335.

Jeevan P, Ramya K, Edith Rena A 2012. Extracellular biosynthesis of silver nanoparticles by culture supernatant of *Pseudomonas aeruginosa*. *Indian J of Biotechnol* 11: 72-76

Joerger R, Klaus T, Granqvist CG 2000. Biologically produced silver carbon composite materials for optically functional thin-film coatings. *Adv Mater* 12:407–409

Kalimuthu K, Babu RS, Venkataraman D, Bilal M, Gurunathan S 2008. Biosynthesis of silver nanocrystals by *Bacillus licheniformis*. *Coll. Surf*. B 65:150–153

Kasthuri J., Veerapandian S., Rajendiran N., Colloids Surf. B: Biointerfaces 68 2009 55–60.

Klaus T, Joerger R, Olsson E, Granqvist CG 1999. Silver-based crystalline nanoparticles, microbially fabricated. *Proc Natl Acad Sci* USA 96: 13611-13614.

Krutyakov YA, Kudrinskiy AA, Olenin AY, Lisichkin GV (2008) Synthesis and properties of silver nanoparticles: advances and prospects. *Rus Chem Rev* 77:233–257.

Mohamed E, Yousef N, Farag A 2012. Isolation and molecular identification of polyaromatic hydrocarbons–utilizing bacteria from crude petroleum oil samples. *African J microbiol Res* 6:7479-7484.

Morones JR, Elechigurra JC, Camacho A, Holt K, Kouri JB 2005. The bactericidal effect of silver nanoparticles. *Nanotechnology* 16: 23 46-53.

Prasad K, Jha A 2010. Biosynthesis of metal and oxide nanoparticles using Lactobacilli from yoghurt and probiotic spore tablets. *Anaytical J of Biotechnol* 5: 285-291.

Rai M, Yadov A, Gade A 2009. Silver nanoparticles as a new generation of antimicrobials. Biotechnol Advces 27: 76-83

Russel AD, Hugo WB 1994. Antimicrobial activity and action of silver. *Prog. Med. Chem*., 31: 351-370.

Sambrook J, Fritsch E F, Maniatis T 1989. Molecular cloning - A laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press.

Saravanan M, Vemu AK, Barik SK 2011. Rapid biosynthesis of silver nanoparticles from *Bacillus megaterium* (NCIM 2326) and their antibacterial activity on multi drug resistant clinical pathogens. *Coll Surf* B 88:325–331

Sastry M, Patil V, Sainkar SR 1998. Electrostatically controlled diffusion of carboxylic acid derivatized silver colloidal particles in thermally evaporated fatty amine films. *J Phys Chem*. B 102:1404–1410

Shahverdi AR, Fakhimi A, Shahverdi HR, Minanian S 2007. Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against *Staphylococcus aureus* and *Escherichia coli*, Nanomedicine 3: 168-171

Shivaji S, Madhu S, Singh S 2011. Extracellular synthesis of antibacterial silver nanoparticles using psychrophilic bacteria. *Process Biochem*. 46: 1800-1807.

Sondi I & Matijevic E 2003. Homogeneous precipitation by enzyme catalyzed reactions. II. strontium and barium carbonates, *Chemical of Material*, 15 1322-1326.

Sondi I & Salopek-Sondi B 2004. Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria, *J. of Coll. and Interface Sci*, 275 177–182.

Yousef, N. and Nafady, N. 2014. Combining of biological silver nanoparticles with antiseptic agent and their antimicrobial activity. *Int. J. of pure and applied bioscience*, 2 (2) 39-47.

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