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Original Research

Antimicrobial Activity of Silver Nanoparticles Synthesized by Marine *Lactobacillus Sp* against Multiple Drug Resistance Pathogens

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Abstract

In this work, *in vitro* biosynthesis of silver nanoparticles was achieved using $AgNO_3$ as a substrate by *L. plantrum* isolated from mangrove rhizosphere region in South East Coast of India (Gulf of Mannar). The biosynthesis was faster within a minute of silver ion coming in contact with the cell filtrate. Presence of silver nanoparticles in the culture filtrate was confirmed by absorption peak at 430 nm. The biosynthesis of nanoparticles was the maximum when the culture filtrate was treated with 1.0mM $AgNO_3$ and pH 6.0, incubated at 5 ◦C for 24 h and its nanoparticles was used for antimicrobial activity. The culture filtrate, precipitated with ammonium sulphate, was proved to have a single protein band with a molecular weight of 70 kDa using polyacrylamide gel electrophoresis. The present work highlighted the possibility of using the marine bacterial strain of *L. plantrum* to achieve a fast rate of nanoparticles synthesis and also used as antimicrobial agent.

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INTRODUCTION

Nanoscience is the study of nanoparticles of 1-100 nanometer size. One nanometer is one billionth of a meter. Nanotechnology is the application of nanoscience, engineering and technology to produce novel materials and devises. Nanotechnology literally means any technology performed on a nanoscale that has application. It encompasses the production and application of physical, chemical, biological systems at scales ranging from individual atoms or molecular to sub-micron dimensions as well as the integration of the resulting nanostructures into larger systems (Feymann, 1960). It is a sub-set of nanotechnology: Atom level engineering and manufacturing using biological precedents for guidance. It is also closely married to Biotechnology but adds the ability to design and modify the atomic – level details of the object created. Bio-nanomachines are designed to atomic specifications, they perform a well defined three – dimensional molecular task, and in the best applications, they contain mechanisms for individual control embedded in their structures.

Recent studies of microorganisms in the synthesis of nanoparticles are a new and exciting area of research with considerable potential for development (Deendayal *et al.,* 2006). Bacteria are known to enrich ions and synthesize magnetite crystals (Beveridge *et al.,* 1980; Beveridge *et al.,* 1997) and to convert metals into nanoparticles of silver and gold (Gericke and Pinches, 2006; Kalimuthu *et al.,* 2008). The use of fungi and bacteria in the synthesis of nanoparticles is potentially exciting since they secrete large amounts of enzymes and are simpler to deal within the laboratory (Deendayal *et al.,* 2006; Mukherjee *et al.,* 2002, Kuber and D'Souza, 2006). Nanotechnology detects human disease such as Human immunodeficiency virus (HIV), Malaria, Tuberculosis (TB), Diabetics, and heart problems. CSIO (Council of Scientific and Instrument Organization-India), developed a micro diagnostic kit using monosized biosensors like Antibodies, Antigen and DNA. Hence, the present study was undertaken to prove the potential in extra-cellular biosynthesis of silver nanoparticles and antimicrobial activity by a bacteria of plant origin.

MATERIALS AND METHODS

Microorganism

The marine bacteria, *L. plantrum* were isolated from the rhizosphere soil was used in this study. It was sub-cultured in Modified MRS agar medium.

Biomass Production

To prepare biomass for biosynthesis studies, the sub - cultured marine bacteria, *L. plantrum* was grown aerobically in a liquid media containing (g/l) KH_2PO_4–7.0; K_2HPO_4–2.0; $MgSO_4$–0.1; $(NH_4)_2SO_4$–1.0; yeast extract–0.6; and glucose–10.0. The flasks were inoculated and incubated on orbital shaker at 25 ◦C and agitated at 150 rpm. The biomass was harvested after 72 h of growth by sieving through a plastic sieve, followed by extensive washing with distilled water to remove any medium component from the biomass.

Silver Nanoparticles Synthesis Using Silver Nitrate

Typically 20 g of fresh biomass was brought in contact with 200ml of Milli-Q deionized water for 72 h at 25 ◦C in an Erlenmeyer flask and agitated in the same condition as described earlier. After incubation, the cell filtrate was obtained by passing it through Whatmann filter paper no. 1. For synthesis of silver nanoparticles, $AgNO_3$–1mM was mixed with 50 ml of cell filtrate in a 250 ml Erlenmeyer flask and agitated at 25 ◦C in dark. Control (without the silver ion, only biomass) was also run along with the experimental flask. Sample of 1ml was withdrawn at different time intervals and the absorbance was measured at a resolution of 1 nm using a UV–visible Spectrophotometer (Elico, Chennai).

Absorbance Spectra of Nanoparticles

To find out the absorbance peak of Nanoparticles 1mM (final concentration) $AgNO_3$ was mixed with 50 ml of cell filtrate in a 250 ml Erlenmeyer flask and agitated in dark. Then after 24 h, optical density was taken at different wavelength ranging from 300 to 700nm and plotted the values on a graph.

Optimization of Physio–Chemical Parameters for Biosynthesis of Nanoparticles

Influence of temperature, pH, sources of carbon and nitrogen on the production of silver Nanoparticles were optimized by varying the parameters one at a time, such as temperature (0, 5, 20, 30, 40 ◦C), pH (5.0, 6.0, 7.0, 8.0 and 9.0), substrate concentration (0.5, 1, 1.5, 2.0, 2.5mM $AgNO_3$) and incubation period (0-48 h). Sample of 1ml was withdrawn at different time intervals and the absorbance was measured at 430 nm.

Antimicrobial activity of $AgNO_3$ Nanoparticles against MDR Pathogens

The antimicrobial activity of $AgNO_3$ nanoparticles was evaluated against different kinds of MDR pathogens by modification of the agar disc diffusion methods. The each MDR pathogen were inoculated on nutrient agar plates and Muller Hinton agar plates was using, and then 20 µL of $AgNO_3$ nanoparticles disc were placed into the plates. Gentamicin was used as positive controls. The plates were incubated for 24 hours at 37^0C, to evaluate the growth inhibition of $AgNO_3$ nanoparticles. The results on the three plates corresponding to a particular sample were averaged and this value regarded as the zone of inhibition was measured of $AgNO_3$ nanoparticles against MDR pathogens.

Partial Purification and PAGE Analysis

1mM of AgNO3 was mixed with 50 ml of cell filtrate in a 250 ml Erlenmeyer flask and agitated in dark. Then after 24 h, the cell-free culture supernatant was precipitated by using solid ammonium sulphate to 80% saturation. The pellet obtained after centrifugation was dissolved in 0.05M phosphate buffer (pH 8.0). The protein concentration was determined according to Lowry *et al.* (1951) method. The concentrated protein was dialyzed overnight against 0.05M phosphate buffer (pH 8.0) to remove salt. Samples were analyzed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis according to Laemmli (1970) to check the purity and to determine the molecular weight of the purified protein by comparing with protein standard having molecular weight ranging from 10 to 250 kDa (Lonza marker).

RESULTS

The marine bacterial strains of *L. plantrum* culture filtrate exhibited a gradual change in colour towards brown when it was incubated with silver ion and maintained under dark. The colour of the culture filtrate was changed to intense brown after 24 h of incubation. Control (without silver ion) did not exhibit any colour change of the culture filtrate.

Nanoparticles synthesis in terms of colour intensity of culture filtrate was examined at different wavelengths (400-500 nm) (Figure 1), pH (5-9) (Figure 3), incubation time (0 min–48 h) (Figure 2), temperature (0, 5, 20, 30, 40 and 50 ◦C) (Figure 4) and silver nitrate (substrate) concentration (0.5–2.5mM) (Figure 5). The absorbance spectra exhibited a peak at 430 nm. At this wavelength, the highest optical density was found at 24 h of incubation time, pH 6.0, temperature of 5 ◦C, at 1.0mM concentration of silver nitrate, and 0.3% sodium chloride.

The nanoparticles was taken for antibacterial screening against two gram +ve and two gram –ve microorganisms and antifungal screening against two different fungal spores i.e. *Candida* sp. The observations revealed that the nanoparticles showed considerable activity against *S. typhi* 35mm and moderate activity in case of *Candida albicans* 26mm. However, very mild activity was observed against gram negative organisms as compared to standard Streptomycin whereas in case of antifungal the extract showed considerable activity against *Candida* sp and moderate activity (Figure 6).

The purification of protein in culture filtrate is shown in Table 1. Protein was purified 1.15 fold. Protein concentration of partially purified preparation was 160g/ml with total recovery of 28.2%. The SDS-PAGE revealed a single protein band of culture filtrate with molecular weight of 70 kDa (Figure 7).

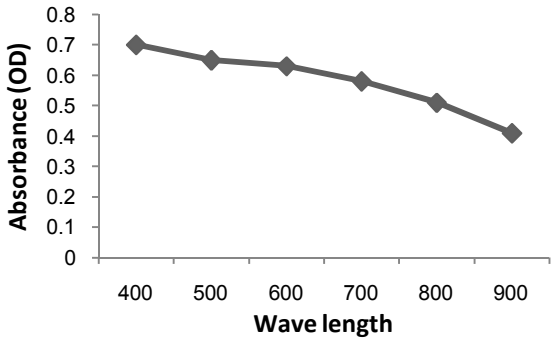

Figure 1: Absorption spectrum for silver nanoparticles synthesis at long wavelength.

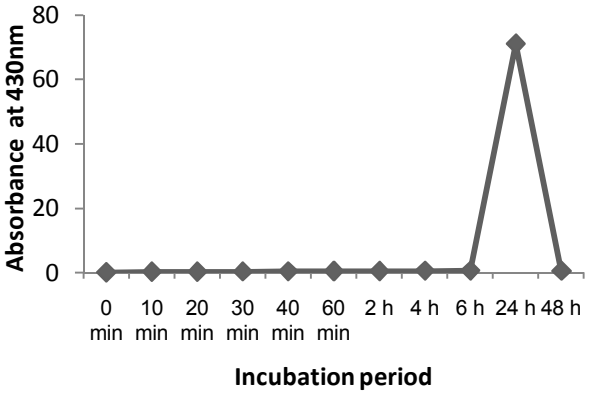

Figure 2: Effect of incubation period on silver nanoparticle synthesis by *Lactobacillus plantrum.*

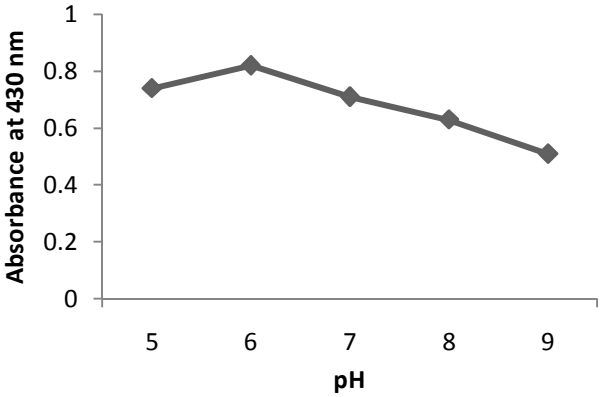

Figure 3: Effect of pH on silver nanoparticle synthesis by *Lactobacillus plantrum*

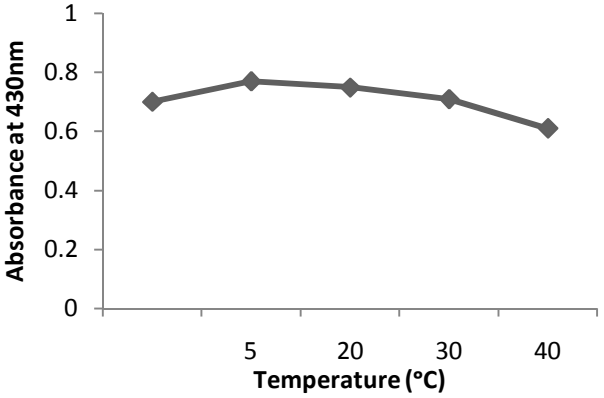

Figure 4: Effect of temperature on silver nanoparticle synthesis by *Lactobacillus plantrum*

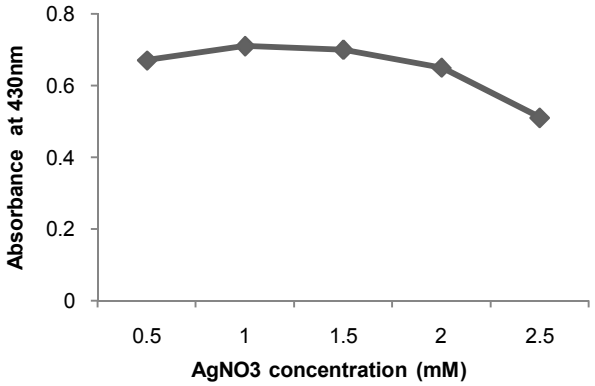

Figure 5: Effect of substrate ($AgNO_3$) concentration on silver nanoparticle synthesis by *L. plantrum*

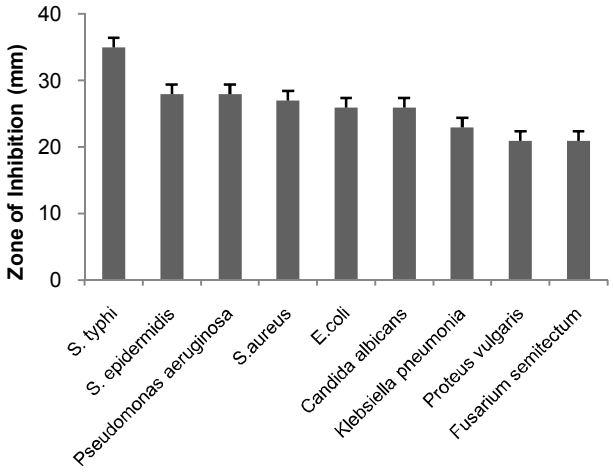

Figure 6: Antimicrobial activity of silver nanoparticles synthesis by *L. plantrum* against MDR pathogens.

Table 1: Purification scheme of silver nitrate added culture filtrate of *L.plantrum*

Purification steps	Quantity (ml)	Protein (µg/ml)	Total protein (mg)	Yield (%)	Purific-ation fold
Crude extract	100	83	8.3	100	1.00
Ammonium sulfate(40%)	25	120	3.0	35	1.06
Ammonium sulfate(80%)	15	160	2.3	28.1	1.14

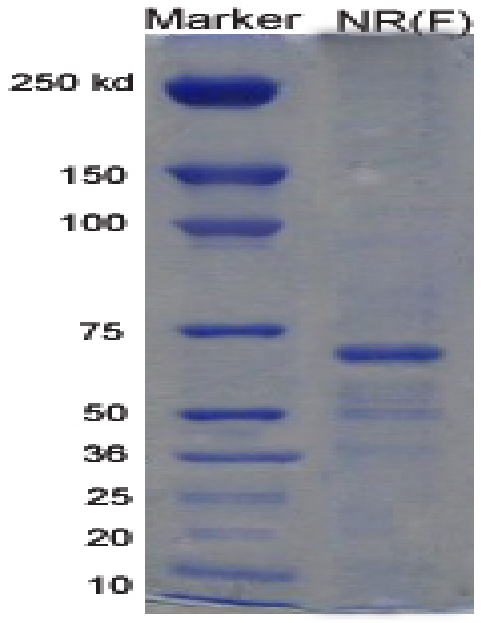

Figure 7: SDS-PAGE patterns of purified protein of silver nitrate.

DISCUSSION

Silver nanoparticles have applications in spectrally selective coating for solar energy absorption, optimal receptors in intercalation material for electrical batteries,

polarizing filters, catalysts in chemical reaction, bio-labeling and as antimicrobial agents (Kuber and D'Souza, 2006). These applications are dependent on synthesized silver particles and their chemical stability without undergoing degradation like partial oxidation. There are several physical and chemical methods for synthesis of metallic nanoparticles (Kowshik *et al.,* 2003). However, biological methods may be relatively simple, reliable, eco-friendly and promising. In this regard, microorganisms such as bacteria, fungi and yeast are known for their ability to reduce metal ions to form metallic nanoparticles (Deendayal *et al.,* 2006). There are only few papers concerned with silver nanoparticles synthesis by fungi that too confined to terrestrial strains (Kowshik *et al.,* 2003). The present study proved an extra-cellular biosynthesis of silver nanoparticles by a marine *Lactobacillus* sp isolated from root-soil. It was observed that upon addition of the silver ion (1mM) into the flask containing the culture filtrate, the colour of the medium got changed very rapidly to brown. This indicated the presence of silver nanoparticles which could be due to the excitation of surface plasmon vibrations, typical of the silver nanoparticles (Deendayal *et al.,* 2006). The silver nanoparticles were synthesized within 10 min of silver ions coming in contact with the culture filtrate**.** This is the second report after Kuber and D'Souza (Kuber and D'Souza, 2006) displaying such a rapid biosynthesis of nanoparticles within minutes. In another report, Deendayal *et al.* (2006) have shown extra-cellular synthesis of silver nanoparticles within hours. The increase in colour intensity of culture filtrate was due to increasing number of nanoparticles formed as a result of reduction of silver ions present in the aqueous solution. The fact that silver nanoparticles peak remained close to 430nm even after 24 h of incubation (Figure 2) indicates that the particles are well-dispersed in the solution and this monodispersity is an important characteristic of the nanoparticles. Silver nanoparticles synthesized by using *Fusarium oxysporum* are reportedly having very good monodispersity as well stability for even up to 4 months of incubation at 25 ◦C (Deendayal *et al.,* 2006) and these are favourable characters for potential application of nanoparticles (Deendayal *et al.,* 2006).

In earlier studies on the synthesis of silver and gold nanoparticles using bacteria (Klaus *et al.,* 1999) and fungi (Mukherjee *et al.,* 2001), the time required for completion of the reaction (i.e., complete reduction of the metal ions) ranges from 24 to 120 h, while the 24 h incubation exhibited maximum synthesis of silver nanoparticles. Metal accumulation is dependent on the growth phase of cells (Mukherjee *et al.,* 2002). In *Desulfovibrio desulfuricans*, reduction of palladium ions is accomplished within minutes, but it is not a purely biosynthetic process, since an exogenous electron donor is needed to accomplish the reduction process (Mehra and Winge, 1991). In the present study, pH6.0 supported the maximum synthesis of silver nanoparticles (Figure 3) whereas optimum gold accumulation by microbial cells normally occurs in the pH range of 2–6 (Joerger *et al.,* 2000) and changes in the pH has an effect on the size of gold nanoparticles (Mukherjee *et al.,* 2001).

Application of the biological systems for synthesis of silver nanoparticles is well known (Klaus *et al.,* 1999). However, exact reaction mechanism leading to the biosynthesis of silver nanoparticles is yet to be elucidated. NADH-dependent reductase is believed to involve in reduction of silver ions in case of *F. oxysporum* (Deendayal *et al.,* 2006).

The antibacterial activity of silver nanoparticles was investigated against various Multi drug resistance pathogenic bacteria and fungal strains of *S. typhi, S. epidermidis, Pseudomonas aeruginosa, S.aureus, S.aureus, E.coli, Candida albicans, Klebsiella pneumonia, Klebsiella pneumonia, Proteus vulgaris* and *Fusarium semitectum* using disc diffusion technique. Control is also maintained in which no zone of inhibition is observed and measured the diameter of inhibition zones around each disc with silver nanoparticles (AgNPs) is represented in Figure 6. Silver has been used for its well known antimicrobial properties since roman time however the advances in generating AgNPs have made possible a revival of the use of silver as a powerful bactericide (Song *et al.,* 2006). Researchers (Feng *et al.,* 2003) have used *Escherichia coli* as a model for gram negative bacteria and proved that AgNPs may be used as an antimicrobial agent. Other workers (Yamanaka *et al.,* 2005) also opined that the AgNPs have an antimicrobial effect on S. aureus and *E. coli.* In the present study, 0.002 mg of the nanoparticles was taken as final product for antimicrobial assay. The antimicrobial activity of the bio-nanoparticles for *S. typhi* was maximum (35 mm) followed by *S. epidermidis* (28 mm), *Pseudomonas aeruginosa* (28 mm), *S. aureus* (27 mm), *E.coli* (26 mm), *Candida albicans* (26 mm), *Xanthomonas citrii* (25 mm), *Klebsiella pneumonia* (23 mm), *Proteus vulgaris* (21 mm), *Fusarium semitectum* (21 mm).

CONCLUSION

In the present study, a single prominent protein band, with low molecular weight of 70 kDa (Figure 7) was detected to be present in the culture filtrate, secreted out of the bacterial biomass and the enzyme protein of nitrate reductase might have involved in the reduction of the silver ions nanoparticles. The present study reported extra-cellular synthesis of silver nanoparticles by a marine bacterium, *L.plantrum* and the process was controlled by pH, temperature, silver ion concentration and exposure time to silver nitrate. Further studies are required on fundamental understanding of mechanism of nanoparticles synthesis at cellular and molecular level.

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REFERENCES

Ahmad, A., Mukherjee, P., Senapati, S., Mandal, D., Khan, M.I., Kumar, R., Sastry, M. (2003). Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium oxysporum*. *Colloids and Surfaces B: Biointerfaces* 28: 313-318.

Beveridge, T.J., Murray, R.G. (1980). Sites of metal deposition in the cell wall of *Bacillus subtilis*. *Journal of Bacteriology* 141(2): 876-887.

Beveridge, T.J., Hughes, M.N., Lee, H., Leung, K.T., Poole, R.K., Savvaidis, I., Silver, S., Trevors, J. T. (1997). Metal-microbe interactions: contemporary approaches. *Advances in Microbial Physiology* 38: 177–243.

Deendayal, M., Bolander, E.M., Mukhopadhyay, D., Sarkar, G., Mukherjee, P. (2006). The use of microorganisms for the formation of metal nanoparticles and their application. *Journal of Biotechnology* 69: 485-492.

Feng, Q.L., Wa, J., Chen, G.Q., Cui, K. Z., Kim, T.M., Kim, J.O. (2003). Antimicrobial activity of silver nanoparticles against bacterial species. *Journal of Biomedical Materials Research* (52): 662-668.

Feynman, Richard P. (1960). There's Plenty of Room at the Bottom. *Engineering and Science* 23 (5): 22-36.

Gericke, M., Pinches, A. (2006). Biological synthesis of metal nanoparticles. *Hydrometallurgy* 83(1-4): 132-140.

Joerger, R., Klaus, T., Granqvist, C.G. (2000). Biologically produced silvercarbon composite materials for optically functional thin-film coatings. *Advanced Materials* 12: 407-409.

Kalimuthu, K., Babu, R.S., Venkataraman, D., Bilal, M., Gurunathan, S. (2008) Biosynthesis of silver nanocrystals by *Bacillus licheniformis*. *Colloids and Surfaces B: Biointerfaces* 65: 150-153.

Klaus, T., Joerger, R., Olsson, E., Granqvist, C.G. (1999). *Proceedings of the National Academy of Sciences* 96: 13611-13614.

Kowshik, M., Ashtaputre, S., Kharrazi, S., Vogel, W., Urban, J., Kulkarni, S.K., Paknikar, K.M. (2003). Extracellular synthesis of silver nanoparticles by a silver-tolerant yeast strain MKY3. *Nanotechnology* 14: 95-100.

Kuber, C.B., D'Souza, S.F. (2006). Extracellular biosynthesis of silver nanoparticles using the fungus *Aspergillus fumigatus*. *Colloids and Surfaces B: Biointerfaces* 47: 160–164.

Laemmli, U.K. (August 1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4". *Nature* 227 (5259): 680–685.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193: 265.

Mehra, R.K., Winge, D.R. (1991). Metal ion resistance in fungi: molecular mechanisms and their regulated expression. *Journal of Cellular Biochemistry* 45(1): 30-40.

Mukherjee, P., Ahmad, A., Mandal, D., Senapati, S., Sainkar, S.R., Khan, M.I., Parischa, R., Ajayakumar, P.V., Alam, M., Kumar, R., Sastry, M. (2001). Fungus mediated synthesis of silver nanoparticles and their immobilization in the mycelial matrix: A novel biological approach to nanoparticle synthesis. *Nano Letters* 1: 515-519.

Mukherjee, P., Senapati, S., Mandal, D., Ahmad, A., Khan, M.I., Kumar, R., Sastry, M. (2002). Extracellular synthesis of gold nanoparticles by the fungus *Fusarium oxysporum*. *European Journal of Chemical Biology* 3: 461–463.

Song, H.Y., Ko, K.K., Oh, I.H., Lee, B.T. (2006). Fabrication of silver nanoparticles and their antimicrobial mechanism. *European Cells and Materials* 11:58.

Yamanaka, M., Hura, K., Kudo, J. (2005). Study of Antibacterial activity. *Applied and Environmental Microbiology* (71): 7589-7593.