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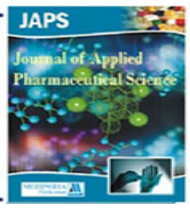

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Antimicrobial Activity of Extracellularly Synthesized Silver Nanoparticles using *Lactobacillus* Species Obtained from VIZYLAC Capsule

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ABSTRACT

The development of green experimental processes for the synthesis of nanoparticles is a need in the field of nanotechnology. In present study deals with the extracellular biosynthesis of silver nanoparticles using VIZYLAC capsule containing about 10^6 *Lactobacillus*. Two different Species of *Lactobacillus* obtained from VIZYLAC capsule were used for the synthesis of silver nanoparticles. Silver nanoparticles were formed by treatment of aqueous solution of $AgNO_3$ (1mM) with supernatant obtained from 24 hour grown culture of *Lactobacillus Spp.* The synthesis of silver nanoparticles was achieved within 3 days of incubation. UV-visible specrophotometric analysis was carried out to assess the formation of silver nanoparticles. Silver nanoparticles were further characterized using Nanoparticle Tracking Analyzer (NTA), Transmission Electron Microscopy (TEM) and Energy Dispersisve X-ray Spectrum (EDX). Synthesized silver nanoparticles showed antibacterial activity against hospital isolate of *Proteus mirabilis* (Multiple Drug Resistent), *Salmonella typhi*, and *Klebsiella pneumoniae*. The antifungal activity was checked against *Candida albicans* (hospital isolate) and *Aspergillus niger* (NCIM 616). The extracellular synthesis of size and shape controlled, monodispersed silver nanoparticles using *Lactobacillus* species appears to be cost effective and eco-friendly.

Keywords: Green synthesis, Nanoparticle Tracking Analyzer, Silver Nanoparticles, Transmission Electron Microscopy,

INTRODUCTION

In recent years, nanotechnology research is emerging as cutting edge technology interdisciplinary with physics, chemistry, biology, material science and medicine. The prefix nano is derived from the Greek word *Nanos* refers to things of one billionth (10^{-9} m) in size (Narayanan *et al.*, 2010). A number of physical and chemical approaches are available for the synthesis of silver nanoparticles (Goia *et al.*, 1998; Vaidyanathan *et al.*, 2009). The chemical and physical methods are harmful in one or the other way, as the chemicals used are toxic, flammable and do not dispose off in the environment easily (Kowshik *et al.*, 2003). These methods lead to presence of toxic chemicals adsorbed on the surface of nanoparticles that may have adverse effect in the medical applications (Jain *et al.*, 2010). Therefore, there is still need for economic, commercially viable as well environmentally clean synthesis route to synthesize silver nanoparticles. Thus, researchers are focusing on green synthesis of silver nanoparticles synthesis.

Various bacteria (Kalimuthu *et al.,* 2008; Saifuddin *et al.,* 2009; Chaudhari *et al.,* 2012) fungi (Vigneshwaran *et al.,* 2007; Gade *et al.,* 2008) and plant sources (Raut *et al.,* 2010; Masurkar *et al.,* 2011) have been used to achieve green synthesis of silver nanoparticles. Green synthesis provides advancement over chemical and physical method as it is cost effective, environment friendly, easily scaled up for large scale synthesis (Deepak *et al.,* 2011).

Previous study on *Lactobacillus* species mediated synthesis of silver nanoparticles indicated that time period required for the synthesis was 2-3 weeks, which is very inconvenient (Karthick *et al.,* 2010). The present article deals with the extracellular synthesis of silver nanoparticles within 3 days using *Lactobacillus* species obtained from VIZYLAC capsule. A VIZYLAC capsule contains about 10^6 *Lactobacillus* was used as source of *Lactobacillus* species. In present article, antibacterial and antifungal activity of silver nanoparticles is also demonstrated.

MATERIALS AND METHODS

Source of *Lactobacillus* species

A VIZYLAC capsule content was dissolved in 0.85% saline under sterile conditions. Loopful of suspension was streaked on sterile *Lactobacillus* MRS Agar plate (Proteose peptone 10 gm, Beef extract 10 gm/L, Yeast extract 5 gm/L, Dextrose 20 gm/L, Polysorbate 80 1 gm/L, Ammonium citrate 2 gm/L, Sodium acetate 5 gm/L, Magnesium sulphate 0.10 gm/L, Manganese sulphate 0.05 gm/L, Dipottasium phosphate 2 gm/L, Agar 12 gm/L, pH 6.5 ± 0.2). The plate was incubated in anaerobic chamber at 37°C for 48 hrs. After incubation, two different types of colonies (named as MRS 1 and MRS 2) were observed. Morphological and Biochemical characterization was done to ensure both the colonies belonged to *Lactobacillus* species.

Collection of supernatant

MRS 1 and MRS 2 colonies were separately inoculated in 0.85% saline and streaked on different sterile *Lactobacillus* MRS Agar plate. The plates were incubated in anaerobic chamber at 37°C for 48 hrs. Single isolated colony of both the *Lactobacillus* strains was inoculated in sterile nutrient broth separately and incubated for 30 hrs at 37º C at 200 rpm. After incubation, the cultures were centrifuged at 4500 rpm for 10 minutes. The supernatants of both the cultures were collected in sterile reagent bottles separately and stored at 4º C. These supernatants were further used as starting material for silver nanoparticles synthesis.

Synthesis of Silver Nanoparticles using culture supernatant of *Lactobacillus* species

1 mM silver nitrate was used as precursor for silver nanoparticles synthesis. Supernatant of both the *Lactobacillus* species was mixed separately with 1 mM silver nitrate in 1:1 ratio. The final pH of the reaction mixture was adjusted to 8.5 (Darroudi *et al.,* 2010; Deepak *et al.,* 2011). The resultant solutions were kept in shaking conditions (200 rpm) at 37° C till colour change was observed.

UV-visible spectrophotometer analysis

After observing colour change, the sample was subjected to mild sonication for 10 minutes. The sample was subjected to scan (300 nm – 600 nm) using Thermo- Biomate 3 UV-visible spectrophotometer. Distilled water was taken to adjust the baseline.

Nanoparticle Tracking Analyzer (NTA) Measurements

NTA analysis was carried out by using Nanosight-LM20 instrument. 0.3 ml sample was introduced to the viewing unit using a disposable syringe and enhanced by a near perfect black background; particles appeared individually as point-scatterers moving under Brownian motion.

Transmission Electron Microscopy (TEM) and Energy Dispersive X-ray Spectra (EDX) analysis

A drop of synthesized nanoparticles solution was placed on carbon coated copper grid and later exposed to infrared light (45 minutes) for solvent evaporation. TEM analysis was performed by using PHILIPS CM 200 instrument operated at an accelerating voltage of 200 kV with resolution of 0.23 nm. The EDX analysis was carried out using JEOL JSM 7600F.

Antibacterial Studies

The antibacterial activity of silver nanoparticles was studied against multiple drug resistant hospital isolate of *P.mirabilis* and hospital isolates of *S.typhi*, *K.pneumoniae*, *C.albicans* (obtained from Department of Microbiology, Pravara Institute of Medical Sciences, Loni, India) and *A.niger* (NCIM 616). The well diffusion test was performed using sterile Muller Hinton Agar no 2. (Casein acid hydrolysate 17.50 gm, Beef heart infusion 2 gm, Starch soluble 1.5 gm, Agar 17 gm, pH 7.3 ± 0.2). The inoculum used was prepared using sterile Nutrient broth (Peptone 10 gm, Beef extract 10 gm, Sodium chloride 5 gm, pH 7.3) and the tube was incubated at 37°C until the turbidity was achieved up to the 0.5 McFarland standard (usually overnight). For *A.niger* inoculum preparation, spores were collected from 7 days old culture of *A.niger* with the help of sterile nicrome loop dipped in normal saline (0.85% w/v) containing Tween-20 (0.1% v/v, Sigma Chemicals). Spore count of 1×10^6 to 5×10^6 spores/ml was adjusted by haemocytometer count. The Mueller-Hinton agar no. 2 plate was inoculated with 2 ml of inoculum by swabbing over plate. Then, agar was punched with the help of sterile borer to create 6 mm well. 30µl of different concentrations of antibiotics and nanoparticles solution in respective wells was added (Table 1). Sterile distilled water and silver nitrate (1mM) were also added as control. Zone of inhibition was measured after incubation with HiMedia scale. The whole experiment was performed in triplicates.

Table. 1: List of microorganisms used with respective Antibiotics/Antifungals

Microorganisms	Antibiotics/ Antifungals
Proteus mirabilis (MDR)	Streptomycin (50µg/ml), Piperacillin (100µg/ml)
Klebsiella pneumoniae	Gentamicin (10µg/ml), Ampicillin (10µg/ml)
Salmonella typhi	Gentamicin (10µg/ml), Chloramphenicol (20µg/ml)
Candida albicans	Amphotericin B (20µg/ml), Fluconazole (10µg/ml)
Aspergillus niger	Amphotericin B (20µg/ml), Fluconazole (10µg/ml)

RESULTS AND DISCUSSIONS

Characterization of *Lactobacillus* species:

The morphological characterization revealed that the both, MRS 1 and MRS 2, cultures contained gram positive bacilli and non-motile. Biochemical characterization deduced that the both MRS 1 and MRS 2 colonies were *Lactobacillus* species (Table 2).

Table. 2: Results of biochemical characterization confirming *Lactobacillus Spp.*

Test	MRS 1 colony Results	MRS 2 colony results
Indole	-	-
Methyl red	-	-
Voges proskauer	-	-
Citrate	-	-
Glucose	+ (G)	+ (G)
Sucrose	+ (G)	+ (G)
Maltose	+	+
Lactose	+ (G)	+ (G)
Mannitol	+	+
Catalase	-	-
Triple Sugar Iron agar	No H_2S production	No H_2S production

Note: - =negative, + = positive, (G) = gas production

Synthesis of Silver Nanoparticles using culture supernatant of *Lactobacillus* species

In the present study, biological synthesis of silver nanoparticles is primarily confirmed by color change of the reaction mixture from pale yellow to brown clearly indicating the formation of silver nanoparticles (Figure 1). Silver nanoparticles were synthesized using both *Lactobacillus* species, MRS1 and MRS 2. The characteristic brown colour due to the excitation of Plasmon vibrations in the nanoparticles provides a convenient signature of their formation.

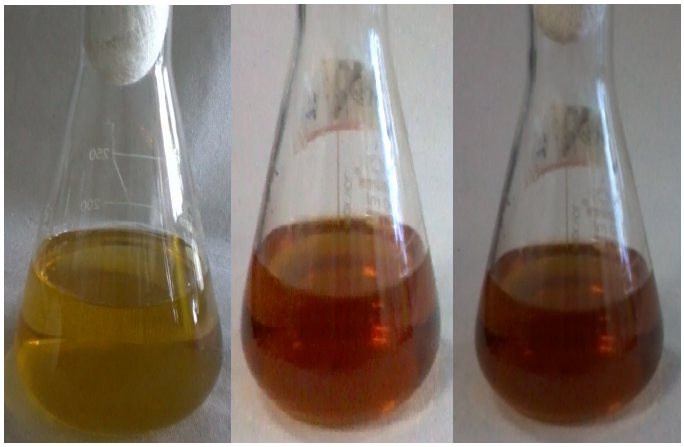


Fig. 1: a. Initial reaction mixture containing *Lactobacillus species* MRS 1 supernatant and 1 mM silver nitrate in 1:1 ratio (pH 8.5); b. Colour change observed of reaction mixture containing *Lactobacillus species* MRS 1 supernatant and 1 mM silver nitrate in 1:1 ratio (pH 8.5); c. Colour change observed of reaction mixture containing *Lactobacillus species* MRS 2 supernatant and 1 mM silver nitrate in 1:1 ratio (pH 8.5).

Earlier, *Lactobacillus* species was used for the synthesis of silver nanoparticles, but the time required for the synthesis was 14-21 days. It was reported that increased pH fastens the rate of synthesis of silver nanoparticles (Deepak *et al.*, 2011). Also, under alkaline conditions the ability of the enzyme responsible for the synthesis of silver nanoparticles increases (Sanghi *et al.*, 2010). In present study, the synthesis of silver nanoparticles was achieved using both *Lactobacillus* species (MRS 1 and MRS 2) supernatant within 3 days on adjusting pH 8.5 of reaction mixtures. After observing colour change, the reaction mixture was centrifuged at 1500 rpm for 5 minutes to remove debris, if any. After centrifugation, the supernatant was collected and was recentrifuged at 13000 rpm for 30-45 minutes. The pellet of silver nanoparticles was suspended in sterile distilled water which was used for further applications.

UV-visible Spectrophotometric analysis

The synthesis of silver nanoparticles can be easily monitored by using UV-visible spectrophotometry. Figure 2 illustrates the absorbance spectra of reaction mixture containing aqueous solution of 1 mM silver nitrate and supernatant of *Lactobacillus* species MRS 1 and the absorbance spectra of reaction mixture containing aqueous solution of 1 mM silver nitrate and supernatant of *Lactobacillus* species MRS 2. Both the reaction mixtures showed an absorbance peak at around 430 nm, which is characteristic of silver nanoparticles, due to its surface plasmon resonance absorption band.

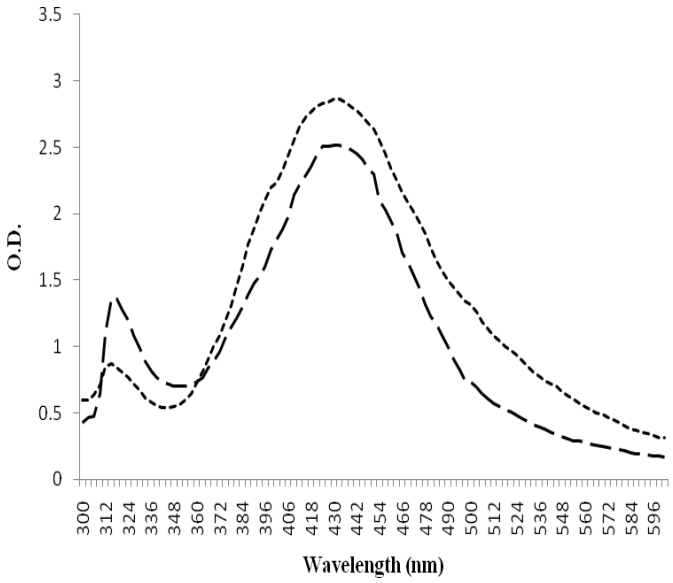
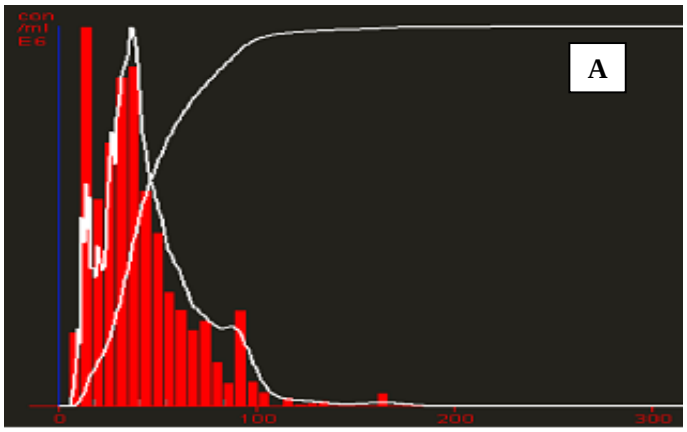


Fig. 2: UV-visible spectrophotometric analysis of synthesized silver nanoparticles.
——silver nanoparticles synthesized using *Lactobacillus* spp. MRS1
------silver nanoparticles synthesized using *Lactobacillus* spp. MRS2

Nanoparticle Tracking Analyzer (NTA) Measurements

NTA measurements revealed that the mean size of silver nanoparticles synthesized using *Lactobacillus* species (MRS 1 and MRS 2) was found to be 41 nm and 39 nm respectively. The concentration of synthesized silver nanoparticles was found to be 3.6×10^{10} particles/ml (for MRS 1 supernatant) and 3.35×10^{10} particles/ml (for MRS 2 supernatant) (Figure 3). The brownian motion of silver nanoparticles was recorded as video clip. No aggregations or debris were detected on NTA measurements, which suggested that the silver nanoparticles suspension is purified.



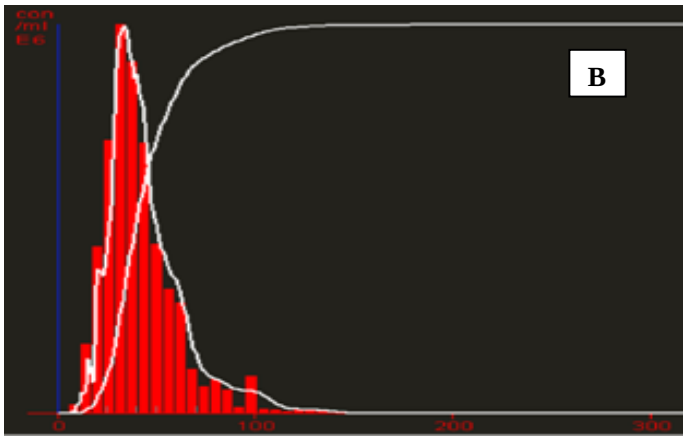
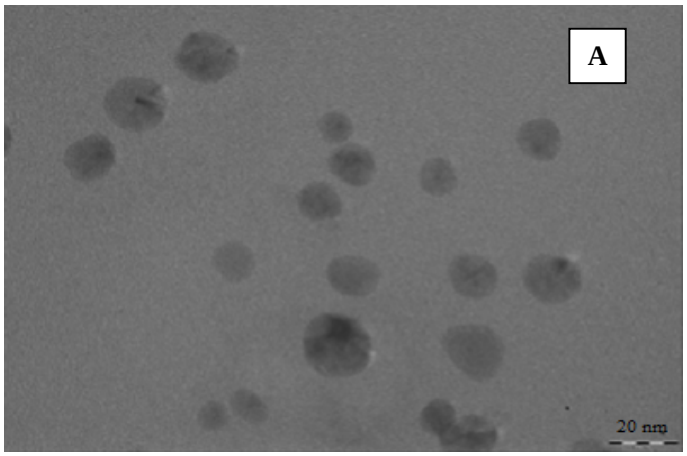


Fig. 3: Size distribution graph of silver nanoparticles synthesized using *Lactobacillus Spp.* (a) MRS 1 supernatant and (b) MRS 2 supernatant. X axis: Particle size in nm; Y axis: Concentration /ml × 10^6.

Transmission Electron Microscopy (TEM) and Energy Dispersive X-ray Spectra (EDX) analysis

TEM analysis revealed that the silver nanoparticles were prominently spherical (Figure 4). The TEM image at high resolution also revealed that silver nanoparticles were not in physical contact but are separated by uniform distance. The capping of silver nanoparticles was also observed under TEM micrograph. The capping may be due to some enzymes or bio-molecules present in the supernatant. The EDX analysis revealed that the silver is present in the solution.



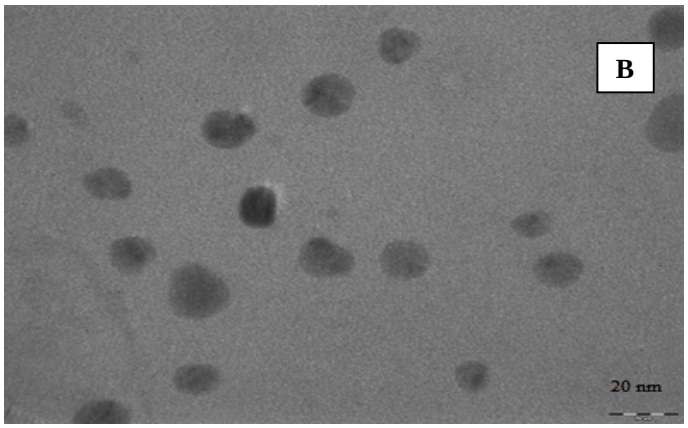


Fig. 4: TEM micrograph of silver nanoparticles synthesized using *Lactobacillus Spp.* (a) MRS 1 supernatant and (b) MRS 2 supernatant.

Antibacterial Studies

The silver nanoparticles showed prominent antibacterial activity against multiple drug resistant hospital isolate of *P.mirabilis*, hospital isolates of *S.typhi*, *K.pneumoniae* and antifungal activity against *C.albicans* (hospital isolate) and *A.niger* (NCIM 616). 1 mM silver nitrate and antibiotics were found to be resistant against tested pathogenic microorganisms (Table 3).

Table. 3: Zone of inhibition (mm) of synthesized silver nanoparticles and antibiotics (or antifungals) against pathogenic microorganisms

Test Microorganisms	Antibiotics (Zone of Inhibition in mm)	Zone of Inhibition in mm		
		Silver nitrate	MRS 1 Silver Nanoparticles	MRS 2 Silver Nanoparticles
P.mirabilis (MDR)	Streptomycin (0) Piperacillin (0)	0	13	14
K.pneumoniae	Gentamicin (0) Ampicillin(0)	0	14	14
S.typhi	Gentamicin (0) Chloramphenicol (0)	0	13	14
C.albicans	Amphotericin B (0) Fluconazole (20)	0	16	16
A.niger	Amphotericin B (0) Fluconazole (0)	0	15	14

CONCLUSION

The present study revealed that the synthesis of silver nanoparticles can be achieved using two different *Lactobacillus* species obtained from VIZYLAC capsule. Previous studies indicated that the 2-3 weeks of time is required for the synthesis of silver nanoparticles using *Lactobacillus* species, but the need in the field of nanotechnology is to synthesize nanoparticles in rapid way without altering its properties. In a present study, synthesis of silver nanoparticles was achieved within 3 days. The rapid synthesis was achieved due to alkaline conditions (pH 8.5) of reaction mixture (Deepak *et al.*, 2011). The synthesized silver nanoparticles were found to be size-shape controlled and well dispersed from each other The synthesized silver nanoparticles were found to be stable even after several months. Synthesized silver nanoparticles showed antimicrobial and antifungal activity against pathogenic microorganisms. This green synthesis approach towards the synthesis of silver nanoparticles has increasing demand in the field of nanotechnology. Applications of such eco-friendly

silver nanoparticles in bactericidal, wound healing and other medical as well as electronic applications, makes this method potentially exciting for the large scale synthesis of nanoparticles. In near future, silver nanoparticles will be used as potential tool to combat against rapidly increasing antibiotic resistance.

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