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Research Article

LACTOBACILLUS SPECIES MEDIATED SYNTHESIS OF SILVER NANOPARTICLES AND THEIR ANTIBACTERIAL ACTIVITY

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ABSTRACT

Silver nanoparticles are among the most commercialized inorganic nanoparticles due to their antimicrobial potential. In the present study the *Lactobacillus* species were isolated from cow milk and their probiotic properties were characterized. The formation of nanoparticles was first screened by measuring the absorbance peak at 430 nm using UV-vis spectroscopy and Fourier Transform Infrared Spectroscopy (FTIR). The morphology of the synthesized AgNPs was determined using TEM, which indicated that the AgNPs were spherical in shape. The synthesized silver nanoparticles (AgNps) showed effective antibacterial activity against bacterial pathogens such as *Eschirichia coli, Staphylococcus auerues, Pseudomonas aeruginosa, Proteus vulgaris, Salmonella typhimurium, Klebsiella pneumoniae* and *Shigella flexneri*. The biosynthesized silver nanoparticles of *Lactobacillus* species showed a very strong inhibitory action against bacterial pathogens and the study supports that AgNPs can be used against gram-negative and gram-positive bacteria for the treatment of infectious diseases.

INTRODUCTION

Probiotic is the group of microbes that may help directly for enhancing resistance against intestinal pathogens and in the prevention of diseases. Probiotic bacteria may produce various compounds, which are inhibitory to the pathogen's growth, which include organic acids (lactic and acetic acids), bacteriocins, and reuterin. The organic acids not only lower the pH, thereby affecting the growth of the pathogen, but they can also be toxic to the microbes. *Lactobacilli* are known to produce many types of bacteriocins like acidophilin, acidolin, lactocidin, bulgarican, lactolin, lactobacillin and lactobrevin. The genus *Lactobacillus* includes rod-shaped bacteria that are generally recognized as safe (GRAS). They have many strains are commercially available as probiotics with health-promoting properties. It belongs to the group of lactic acid bacteria (LAB) (Alvarez-Olmos and Oberhelman, 2001).

Bacteriocins produced by lactic acid bacteria (LAB) are the subject of intense research because of their antibacterial activity against bacteria (Tambekar and Bhutada 2010). Bacteriocins are the biologically active antimicrobial peptides produced by these bacteria and display antimicrobial properties against other bacteria, often closely related to the producer strain (Stern *et al.,* 2006; De Vuyst and Leroy 2007; Tambekar and Bhutada, 2010). These proteinacious compounds have gained great attention and have wide applications (En Yang *et al.,* 2012).

Silver nanoparticles are one of the promising products in the nanotechnology industry. (Azadnia *et al.,* 2011; Awwadi *et al.,* 2012). Ag NPs highly antimicrobial to several species of bacteria, including the common kitchen microbe, *Escherichia coli*. According reported mechanism silver nanoparticles interact with the outer membrane of bacteria, and arrest the respiration and some other metabolic pathway that leads to the death of the bacteria (Baker *et al.,* 2006; Mehrdad and Khalil, 2010; Bankura *et al.,* 2012).

Various physical and chemical methods have been utilized for the production of AgNPs successfully (Bankura *et al.,* 2012; Wei *et al.,* 2012). The synthesis of nanoparticles using conventional physical and chemical methods has a low yield, and it is difficult to prepare AgNPs with a well-defined size. Moreover, chemical methods make use of toxic reducing agents, such as citrate, borohydride, or other organic compounds, and can negatively impact the environment (Medina *et al.,* 2001; Rajeshkumar *et al.,* 2014; Singh 2014).). However, biologically prepared nonmaterial have tremendous

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potential because nanoparticles can easily be coated with lipid/protein/polysaccharides, as well as probably other relatively low-molecular mass compounds that confers physiological solubility and stability, which are critical for biomedical applications and are the bottleneck of other synthesis methods (Paulraj *et al.,* 2013). In the present study silver nanoparticles were synthesized using *Lactobacillus species* and its antibacterial activity was determined against enteric bacterial pathogens.

MATERIALS AND METHODS

Isolation and Identification of Lactobacillus species

Milk samples of domestic Cow were collected randomly in sterilized glass bottles. Milk was serially diluted to 10^{-5} -10^{-6} using sterile distilled water and 0.1mL plated on to sterile de-Mann, Rogosa and Sharpe (MRS) agar. The MRS plates were maintained in microaerophilic condition and incubated at 37°C for 48h. After incubation well-isolated typical colonies were picked up and transferred to MRS broth and incubated at 37°C for 48h. The isolates were identified using standard morphological, cultural and biochemical reactions (Tambekar *et al.,* 2009).

Probiotic Characterization of Isolated Lactobacillus species

Probiotic properties among the identified species were determined through pH tolerance test (pH 3, 4. 5, 7, and 9), bile tolerance test (0.5%, 1.0%, 1.5% and 2.0%) test (Sirilun *et al.,* 2010), and the measurement of antibacterial activity (Todorov *et al.,* 2011). The above tests were carried out in triplicate for each of the isolates.

Bacterial cultures: The standard pathogenic bacterial cultures were procured from IMTECH, Chandigarh, India and used in the present study (Table 1). The bacterial cultures were rejuvenated in Mueller- Hinton broth (Hi-media laboratories, Mumbai, India) at 37^0C for 18h and then stocked at 4^0C in Mueller-Hinton Agar. The inoculum size of the bacterial culture was standardized according to the National committee for Clinical Laboratory Standards (NCCLS, 2002) guideline. The pathogenic bacterial culture was inoculated into sterile Nutrient broth and incubated at 37^0C for 3h until the culture attained a turbidity of 0.5 McFarland units. The final inoculum size was standardized to 10^5 CFU/mL with the help of SPC and Nephlo-turbidometer.

Table 1 Bacterial cultures used in study (IMTECH, Chandigarh, India)

Bacterial Pathogens	MTCC Number
Proteus vulgaris	426
Staphylococcus aureus	96
Escherichia coli	739
Pseudomonas aeruginosa	424
Klebsiella pneumoniae	109
Shigella flexneri	1457
Salmonella typhi	733
Salmonella typhimurium	98

Biosynthesis of Silver Nanoparticles

Lactobacillus species used in the study was isolated from milk sample. The bacteria were identified based on cultural and biochemical characteristics. The isolates were maintained in MRS broth tubes at 4°C. The isolated *Lactobacillus species* was grown in 250-mL Erlenmeyer flasks containing 100 mL - MRS broth at 37°C and 150 rpm for 72 hrs. After incubation, biomass was separated by centrifugation and incubated at 37°C. The filtrate was pale yellow in appearance and the pH was 4.4. A 5 ml of each sample solution taken in a test tube, 1 mg of AgNO3 was added and kept under dark conditions. The primary detection of synthesized silver nanoparticles was carried out in reaction mixture by observing the color change of the medium from pale yellow to reddish brown. After 24hrs of incubation, reaction mixture was centrifuged at 10000 rpm. The suspension was concentrated by repeated centrifugation at 10000 rpm for 10 minutes. The suspension was hot air dried in the oven to make a powder for optical measurements (Dahikar and Bhutada, 2013).

Characterization of Silver Nanoparticle

The bioreduction of Ag ions in aqueous solution was monitored by UV-vis spectra of the solution between 300 nm and 600 nm using UV-vis spectrophotometer (Shimadzu 1650 PC). The nanoparticles scanned the infrared in the region of 750–4000cm^{-1} Fourier transform infrared spectrometer (FTIR, Shimadzu 8400s). The Ag nanoparticle suspension was air-dried on the specimen grid and was observed the micrograph images with Transmission Electron Microscope (TEM).

Antibacterial activity using disc diffusion method: The modified paper disc diffusion (Dahikar and Bhutada, 2013) was employed to determine the antibacterial activity against bacterial on Nutrient agar media. The inhibitory growth effect of various concentrations of silver nanoparticles (50 µg, 100 µg, 150 µg, 200 µg and 250µg). Bacterial cell filtrate used for the synthesis of silver nanoparticles was used as negative control. Ampicillin 10 mcg/disc (Hi-Media, Mumbai) were used as positive control. Turbidity of inoculums was matched with McFarland turbidity standard. Inoculums were spread over the Nutrient agar plate using a sterile cotton swab in order to get a uniform microbial growth. Then the prepared antibacterial disc were placed over the lawn and pressed slightly along with positive and negative controls. The plates were incubated for 18h at 37^0C. The antibacterial activity was evaluated and diameters of inhibition zones were measured. Experiment was carried out in triplicate and the averages diameter of zone of inhibition was recorded. The antibacterial activity was classified as strong (>20mm), moderate (16-19mm) and mild (12-15mm) and less than 12mm was taken as inactive (Bhutada *et al.,* 2011).

RESULTS AND DISCUSSION

Synthesized silver nanoparticles were preliminarily confirmed by the change of color of the solution. The color change of yellow to brown color of the aqueous solution exhibits due to the excitation of the surface plasmon resonance. The color changes from yellow to dark brown indicating the formation of silver nanoparticles at 24 hrs of incubation. After 72 hrs subjected to optical measurements by UV-Vis spectrophotometer, this analysis showed an absorbance peak at 430 nm (Fig.1) which was specific for the silver nanoparticles. Appearance of this absorption shoulder together with hump at 430 nm indicates the presence of nanocrystallites with different sizes.

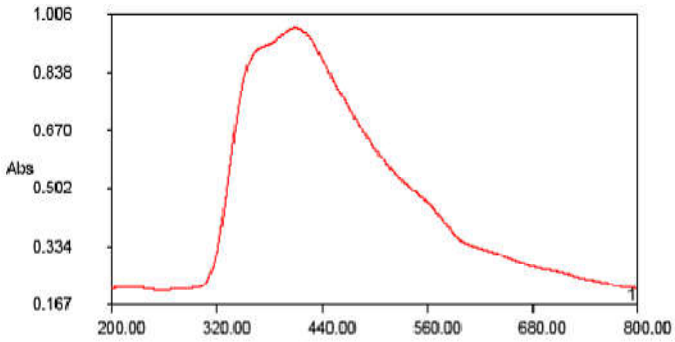

Fig 1 Absorption spectra of silver nanoparticles by UV-visible spectroscopy

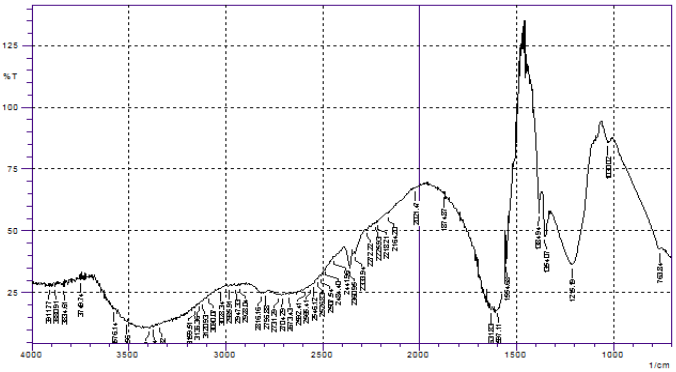

Fig 2 FTIR Spectrum of Silver nanoparticles by using *Lactobacillus sp.*

FTIR shows the biomolecules associated with silver nanoparticles which are responsible for reduction of silver ions to silver nanoparticles. This FTIR spectrum supports the presence of proteins in Ag-NPs showed the presence of bands 3090.07 - NH stretching, 1554.68 - CN Stretching, NH bending, 1631.83 - C=O Stretching, 763.84 - out of plane NH bending.

Transmission Electron Microscopy

Morphological structure and distribution of synthesized silver nanoparticle were characterized at high magnifications done by TEM.

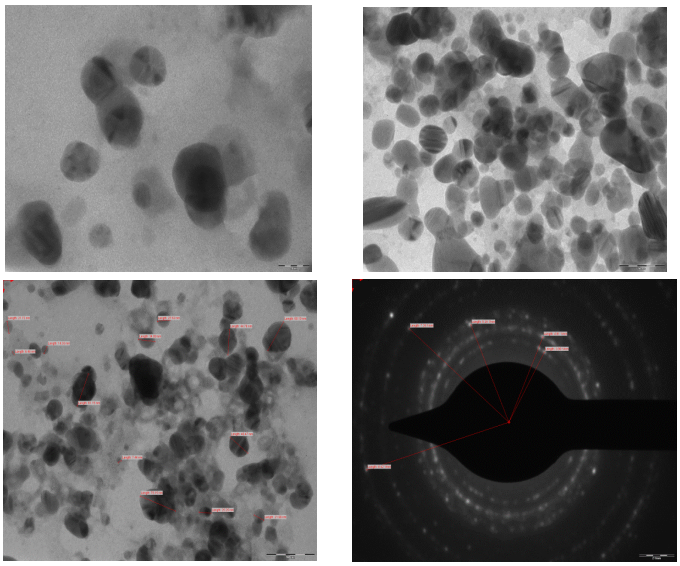

Fig 3 TEM Images of silver nanoparticles of *Lactobacillus sp.*

Transmission Electron Microscopy (TEM) was performed by PHILIPS having Model no. CM-200 (20-200kv) with a line resolution 2.4 A^o , the ample were analysed at IIT, Mumbai. TEM images are formed using transmitted electrons (instead of the visible light) which can produce magnification details up to 1,000,000X with resolution better than 10 A^o.

Antibacterial activity of synthesized silver nanoparticles of Lactobacillus species

The present report showed a rapid and cost-effectiveness method to synthesize silver nanoparticles from *Lactobacillus* species. The zone of inhibition clearly shows that the pathogenic strains tested are responsible for silver nanoparticles. The current report proved that the biologically silver nanoparticles seem to present potential and effective bactericidal covering material.

Table 2 The antibacterial activity of silver nanoparticles of *Lactobacillus species* showed different inhibitory effect on different pathogenic bacteria (Zone of Inhibition in mm)

Bacterial Pathogens	Concentration of AgNp (µg/disc)				
	50	100	150	200	250
E. coli	18	22	24	26	28
S. aureus	17	19	22	24	26
P. aeureginosa	20	23	26	27	28
P. vulgaris	16	18	22	23	25
Sal.typhimurium	15	17	19	22	24
K. pneumoniae	14	17	20	22	23
S. flexneri	16	18	21	24	26
S. typhi	15	17	18	21	25

The antibacterial activity of silver nanoparticles of *Lactobacillus species* showed different inhibitory effect on different human pathogenic bacteria like *Eschirichia coli, Staphylococcus auerues, Pseudomonas aeruginosa, Proteus vulgaris, Salmonella typhimurium, Klebsiella pneumoniae* and *Shigella flexneri,* which showed different inhibition zone with different size. The results are shown into the table which revealed that most of the silver nanoparticles showed antimicrobial activity with varying magnitudes. This dissimilarity might be due to different interactions of nanoparticles with the tested organisms.

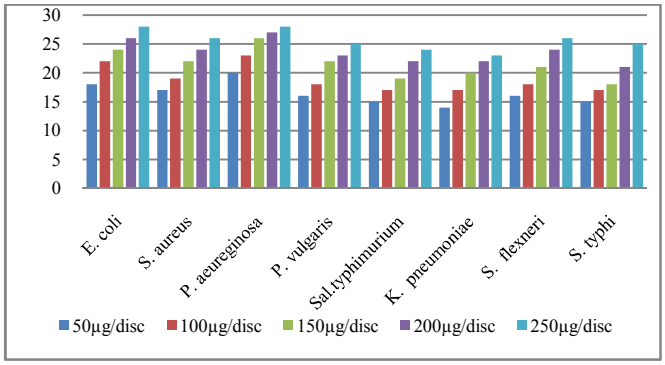

Fig 4 The antibacterial activity of silver nanoparticles of *Lactobacillus* species

CONCLUSION

Probiotic bacteria may produce various compounds, which are inhibitory to the bacterial pathogen. The biosynthesized silver nanoparticles of *Lactobacillus* species showed a very strong inhibitory action against bacterial pathogens and the study supports that AgNPs can be used against gram-negative and gram-positive bacteria for the treatment of infectious diseases.

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