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PRODUCTION, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF SILVER NANOPARTICLES PRODUCED BY *LACTOBACILLUS AMYLOPHILUS* GV6.

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ABSTRACT

Biological production of nanoparticles and their applications is a relatively new area of research. Silver nanoparticles are produced chemically which involves different solvents and are not safe. Production of silver nanoparticles by microorganisms / biological methods is gaining importance as it is safer and useful in both medical and non-medical applications. *Lactobacillus amylophilus* GV6, a GRAS strain is observed to produce silver nanoparticles in fermented broth. We have observed that the strain has ability to produce monodispersed silver nanoparticles in the range of 20 - 25 nm as observed in the TEM micrographs and were characterised by UV-Visible spectrophotometry, AAS, FTIR and XRD. Silver nanoparticles produced by *L. amylophilus* GV6 have antibacterial activity against both Gram positive and Gram negative pathogenic bacteria.

KEYWORDS: Nanoparticles, AgNPs, *L. amylophilus*, antibacterial.

INTRODUCTION

"Silver nanoparticles (AgNPs)" are nanoparticles of silver which are in the range of 1 and 100 nm in size. Silver nanoparticles have unique properties which help in molecular diagnostics, therapies, as well as in devices that are used in several medical procedures. Physical and chemical methods are generally employed for synthesis of silver nanoparticles. The problem with physical and chemical methods is that the synthesis is expensive and can also have toxic substances adsorbed onto them. To overcome this, the biological process provides a feasible alternative. The major biological systems involved in this are bacteria, fungi and plant extracts. The important applications of silver nanoparticles in the medical field include diagnostics and therapeutics. In most of the therapeutic applications, it is the antimicrobial property that is being majorly explored, though the anti-inflammatory property has its fair share of applications.[1] Use of generally recognised as safe (GRAS) microbes like *Lactobacillus* spp. has revealed a faster, simple and efficient method for synthesis of silver nanoparticles. The exact mechanism by which silver nanoparticles cause antimicrobial effect is not clearly known and is a debated topic.[1] There are however various theories on the action of silver nanoparticles on microbes to cause the antimicrobial effect. Silver nanoparticles have the ability to anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes like the permeability of cell membrane and death of the cell. There is formation of 'pits' on the cell surface and also accumulation of nanoparticles on cell surface.[2] The formation of free radicals by the silver nanoparticles may be considered to be another mechanism by which the cells are killed.[3,4] Some bacteria are known to absorb heavy metals from contaminated wastewaters. Some microorganisms not only have a strong biosorptive capacity but also can reduce and precipitate them in their metallic form.[5] This ability of bacteria has unleashed the potential use of biomass in production of nanoparticles. Among the various nanoparticles, metal nanoparticles assume special importance because they are easier and cheaper to synthesize and are the most promising in applications.[6] A number of recent achievements offer the possibility of generating new types of nanostructured materials with designed surface and structural properties.[7-11] The preparation of uniform nanosized drug particles with specific requirements in terms of size, shape, physical and chemical properties is of great interest in the formulation of new pharmaceutical products[11-15] which can be achieved using GRAS microbes like *Lactobacillus*. Biologically synthesized nanoparticles have many applications, such as spectrally selective coatings for solar energy absorption and intercalation material for electrical batteries[16], as optical receptors[17], catalysts in chemical reactions, biolabelling.[18] etc. As a result, researchers in the field of nanoparticle synthesis have turned to biological systems for inspiration.[19,20] Various modes of biosynthesis of silver nanoparticles for development of simple, fast and efficient process that can be scaled up easily are being developed. Many researchers have reported use of plant

extracts and fungi for biological production of silver nanoparticles, though the time taken for production is variable depending on the type of biosynthesis. It is known that a large number of organisms, both unicellular and multicellular are able to produce inorganic nanomaterials, either intracellularly or extracellularly.[21,22] Localization and distribution inside the cell are dependent on species of *Lactobacillus*. *L. fermentum* produced two fractions of the silver nanoparticles. The most prevalent fraction consisted of small particles (5–15 nm) situated in the outer layers of the cell and on the cell wall. In contrast, the less prevalent fraction of silver nanoparticles, ranging from 15 to 40 nm, is intracellular and evenly distributed in the cytoplasm. The nanoparticles produced by *L. plantarum* LMG 24830, *L. plantarum* LMG 24832, *L. farciminis and L. rhamnosus* were situated more frequently inside the cytoplasm.[5] A GRAS microbe that can synthesise silver nanoparticles both intracellularly and extracellulary through a simple process at a faster rate and that can be used for scaleup could be of significance. In the present work we report the ability of *Lactobacillus amylophilus* GV6 to produce monodispersed silver nanoparticles in the range of 25-30 nm which could be of use in drug delivery applications.

MATERIALS AND METHODS

Production and detection of silver nanoparticles

L. amylophilus GV6 was grown in MRS medium for 24 hrs. To the fermented broth, silver nitrate was added at concentrations of 3, 5 and 7 mM. Uninoculated MRS medium with same concentrations of silver nitrate were used as controls. Aliquots of these reaction solutions were collected at intervals of 2, 4 and 6 hrs. Spectrum scan was performed to check the formation of silver nanoparticles using a UV-Visible spectrophotometer (Hitachi U2900) in wavelength range of 190nm – 1100 nm at ambient temperature in solution form.[22]

Characterization of silver nanoparticles

Silver nanoparticles formation was monitored visually and by recording the UV-visible spectrum periodically in a Hitachi U2900 spectrophotometer. The aqueous filtrate containing silver nanoparticles and their controls were subjected to Fourier transform infrared spectrum[23] on Shimadzu FTIR 8400S. The powder obtained after drying of nanoparticles was subjected to X-ray diffraction study using Shimadzu X-ray diffractometer 7000. Powder X-ray diffraction technique was used to determine phase purity, crystal structure and to roughly estimate the size of the nanocrystallites, calculated from the XRD pattern using the Debye-Scherrer formula.[24] Amounts of ionic silver in silver nanoparticles suspensions were measured using a Shimadzu AA 6300 atomic absorption spectrophotometer.[25] Sizes of the nanoparticles were measured by Transmission electron microscopic studies.[24]

Antimicrobial activity of nanoparticles

Silver nanoparticles produced were suspended in deionized water and tested for their antibacterial activity by agar well assay. Five bacterial strains, *Pseudomonas aeruginosa* MTCC424, *Bacillus subtilis* MTCC121, *Staphylococcus aureus* MTCC 96, *E.coli* MTCC43 and *Klebsiella pneumonia* MTCC109, procured from Microbial Type Culture Collection (MTCC), Chandigarh, India were used for this analysis. These bacterial strains were grown in nutrient broth medium for 24 hrs prior to the experiment, seeded in agar plates by the pour plate technique. Inoculum of 10^8 CFU/ml was used for seeding the bacterial cultures. Agar wells were made using a cork borer (5 mm diameter) at an equal distance. A volume of 25 uL of silver nanoparticle solutions produced at three concentrations of silver nitrate (3, 5 and 7 mM) was added to each well and then incubated at 37°C for 24 hrs. A well with only fermented broth containing lactic acid was used as a control. Antimicrobial activity was assessed with silver nanoparticles produced from fermented broth before and after extraction of lactic acid.

Extraction of lactic acid

Lactic acid was extracted by adding excess of calcium carbonate to fermented broth to form calcium lactate. The solution was then centrifuged and the supernatant obtained was checked for presence of lactic acid by spectrophotometry.[26] This supernatant was added with silver nitrate to produce silver nanoparticles.

All the experiments were performed thrice in triplicates at three different instances.

RESULTS

L.amylophilus GV6 fermented broth was observed to reduce silver and produce silver nanoparticles. The colour of the reaction was observed to change from light yellow to brown after 6 hrs of reaction (Fig. 1). Nanoparticles formation is analysed by different methods but a simple method for identification of nanoparticles is by spectrophotometry. Silver nanoparticles exhibit yellowish brown colour in aqueous solution due to excitation of surface plasmon vibrations in silver nanoparticles. Surface Plasmon resonance peak observed at 420 nm (Fig. 2A) is a clear indication of formation of nanoparticles as compared to control (Fig. 2B). Formation of silver nanoparticles were further confirmed by analyzing the characteristic functional groups observed in silver nanoparticles by FTIR analysis (Fig. 3 and Table 1) and XRD pattern (Fig. 4). Bioreduction of silver was analysed and 200 ppm of silver was reduced to 20 ppm ionic silver implying reduction by ten times (Table 2). TEM samples were analyzed on transmission electron microscope and the sizes of the nanoparticles were measured which were observed to be in between 25-50 nm and the particles were observed to be localized both extracellularly (Fig. 5A) and intracellularly (Fig. 5B).

Antimicrobial activity of silver nanoparticles

Silver nanoparticles produced using fermented broth before and after extraction of lactic acid have shown antimicrobial activity against standard bacteria namely *Pseudomonas aeruginosa* MTCC 424, *Bacillus subtilis* MTCC 121, *Staphylococcus aureus* MTCC 96, *E.coli* MTCC 43 and *Klebsiella pneumoniae* MTCC 109 by agar well assay. It was observed that the growth of these bacteria was inhibited from 3mM concentration, and the silver nanoparticles had maximum inhibitory effect against *Pseudomonas aeruginosa* MTCC 424 (2.2 cm) as shown in Fig. 6 and Table 3, followed by *Bacillus subtilis* MTCC 121 (1.6 cm), *Staphylococcus aureus* MTCC 96 (1.5 cm), *E.coli* MTCC 43 (1.2 cm) and *Klebsiella pneumoniae* MTCC 109 (1.2 cm). The control used, which is a fermented broth containing lactic acid (9 mg/ml), showed no zone of inhibition indicating that lactic acid in the broth showed no antibacterial activity. Nanoparticles produced in broth with and without lactic acid have shown same efficacy against the microorganisms tested in this study. Thus making the process more profitable as lactic acid can be extracted first from the fermented broth and then the solution free of lactic acid can be used for production of nanoparticles.

Table1: FTIR analysis of aquaeous filtrate containing silver nanoparticles produced by *L. amylophilus* GV6 for identification of functional groups and comparison with reported characteristic FTIR spectra pattern

Functional group	Characteristic absorption(s) cm^{-1}	FTIR spectrum as observed in the present work
Aromatic C=C bonds	1700 – 1500	1618-1518 cm^{-1}
Ketone C=O stretch	1750 – 1680	1749 -1618
Aldehyde C=O stretch	1740 -1690	1734-1618
Ester C=O stretch	1750 -1735	1749 – 1734
Nitrile C≡N stretch	2260 – 2220	2268 – 2231
Amine N=H stretch	3500 – 3300	3298-3443
Carboxylic acid C=O stretch	3000 – 2500	3005 -2440
Alcohol/Phenol stretch	3550 – 3200	3443 – 3198

Table 2. Bioreduction of Ag^{+} to Ag^{0} by *L.amylophilus* GV6 as measured by atomic absorption spectroscopy

Ag conc. in Control (ppm)	Ag conc. in Test (ppm)
200	**20.0**
200	**20.0**
200	**19.8**

Table 3. Antimicrobial activity of silver nanoparticles produced by *L. amylophilus* GV6 from fermented broth as tested by agar well assay

Test organism	Zone of inhibition (cm) by silver nanoparticles at various conc (mM)		
	3	5	7
Pseudomonas aeruginosa MTCC 424	2.2	2.3	2.4
	2.2	2.3	2.4
Bacillus subtilis MTCC 121	1.6	1.6	1.8
	1.6	1.6	1.8
Staphylococcus aureus MTCC 96	1.5	1.8	1.9
	1.5	1.8	1.9
Escherichia coli MTCC 43	1.2	1.4	1.4
	1.2	1.4	1.4
Klebsiella pneumoniae MTCC 109	1.2	1.2	1.2
	1.2	1.2	1.2

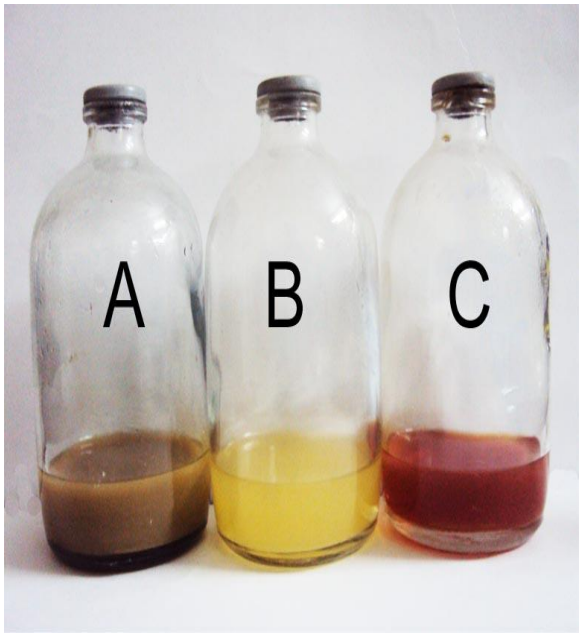

Fig. 1. Production of silver nanoparticles in MRS medium by *L. amylophilus* GV6 as observed by

(A) Change in colour of fermented broth when added with silver nitrate

(B) MRS broth inoculated with organism as growth control

(C) Uninoculated MRS broth added with silver nitrate as negative control

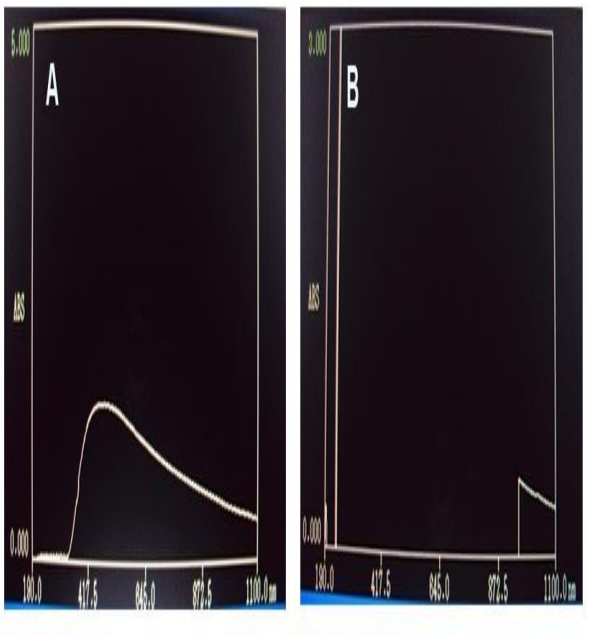

Fig. 2. Wavelength scan of silver nanoparticles produced by *L. amylophilus* GV6 by observing in a UV-visible spectrophotometer

(A) Surface plasmon resonance peak at 420 nm

(B) Uninoculated medium added with silver nitrate as negative control

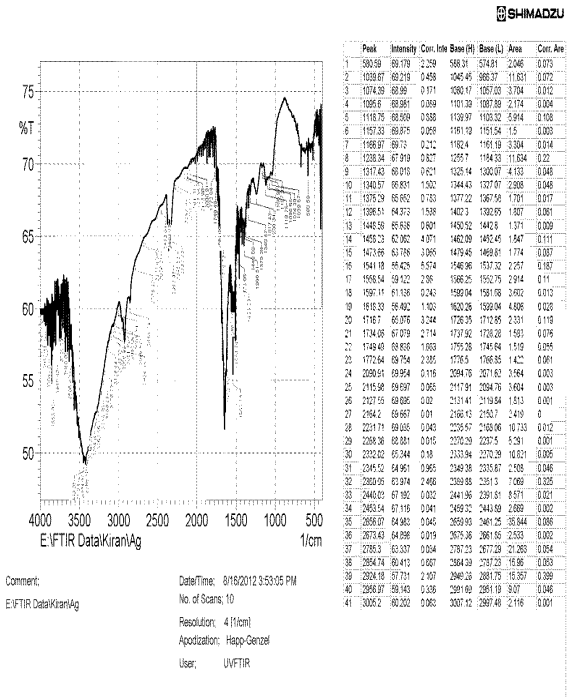

Fig. 3. Aquaeous filtrate with silver nanoparticles was analysed for the functional groups by FTIR spectrum pattern.

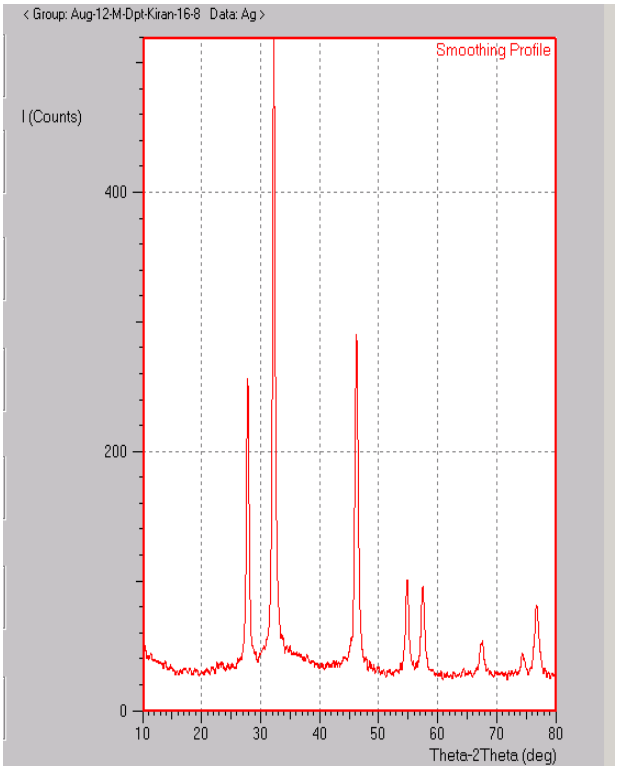

Fig. 4. Silver nanoparticles produced by *L.amylophilus* GV6 was analysed by X-ray diffraction for identifying its nature and size.

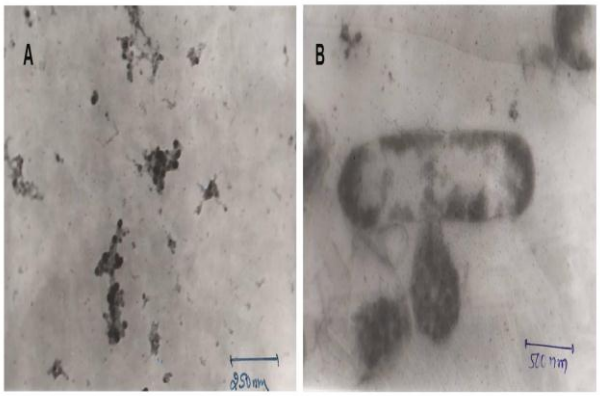

Fig. 5. Transmission electron micrograph of silver nanoparticles produced by *L. amylophilus* GV6 for their size and location - (A) Extracellular and (B) intracellular

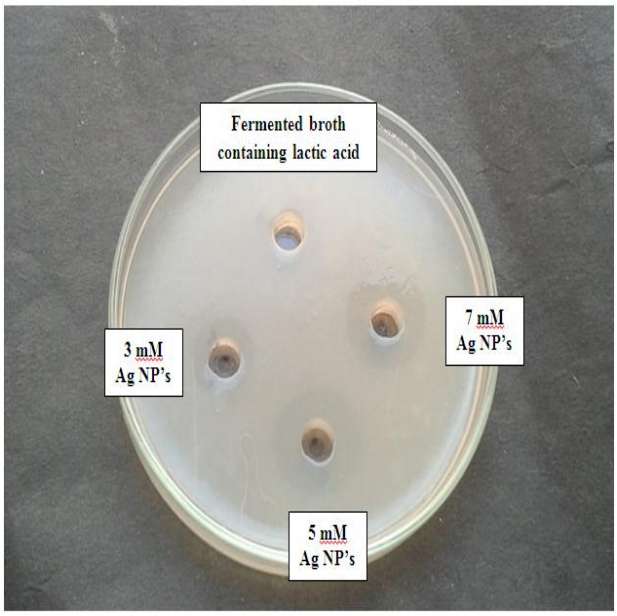

Fig. 6. Antibacterial activity of silver nanoparticles on *Pseudomonas aeruginosa* MTCC 424 by agar well assay method.

DISCUSSION

Nanotechnology research is an emerging area in recent years and is interdisciplinary with physics, chemistry, biology, material science and medicine. The prefix nano is derived from the Greek word refers to things of one billionth (10^{-9} m) in size.[27] A number of physical and chemical approaches are available for the synthesis of silver nanoparticles.[28,29] The chemical and physical methods are harmful in one way or the other, as the chemicals used are toxic, flammable and cannot be disposed off in the environment easily. These methods lead to presence of toxic chemicals adsorbed on the surface of nanoparticles that may have adverse effects in the medical applications.[30] Therefore, there is still need for economic, commercially viable as well environmentally clean route to synthesize silver nanoparticles. Thus, researchers are focusing on green synthesis of silver nanoparticles. Various bacteria[31-33], fungi[34, 35] and plant sources[36, 37] have been used to achieve green synthesis of silver nanoparticles. Green synthesis provides advancement over chemical and physical methods as it is cost effective, environment friendly, easily scaled up for large scale synthesis.[38, 39] *L. amylophilus* GV6 known to be an efficient lactic acid producer is studied for its ability to produce silver nanoparticles by making use of biomass generated after fermentation/fermented broth/broth left over after lactic acid extraction. It has been observed that the production of silver nanoparticles by *L. amylophilus* GV6 is simple and efficient, resulting in production of spherical and uniform silver nanoparticles. When challenged with different concentrations of silver nitrate from 3 - 7 mM, formation of silver nanoparticles was confirmed with the change in color of medium from yellow to brown colour which is due to the surface plasmon resonance (SPR) phenomenon. Production of silver nanoparticles is observed to occur within 6-12 hrs which is much faster than earlier observations reported. Previous study on *Lactobacillus* spp. mediated synthesis of silver nanoparticles took 2-3 weeks[39] and 3 days.[40] Fermented broth, supernatant, bacterial cells were observed to reduce silver and produce silver nanoparticles. Further, fermented broth from which lactic acid is completely extracted was also observed to produce silver nanoparticles. Characterization of silver nanoparticles was performed by different techniques like atomic absorption spectroscopy, X-ray diffraction, fourier transform- infra red spectroscopy, scanning electron microscopy and transmission electron microscopy.

CONCLUSION

1. *Lactobacillus* strains are of importance in production of silver nanoparticles,
2. Few *Lactobacillus* species are observed to tolerate and reduce metals even at higher concentration.
3. *L. amylophilus* GV6 has been observed to possess the ability to reduce metals and produce silver nanoparticles in this study.
4. Apart from its ability to produce lactic acid, its ability to produce nanoparticles is new observation.

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