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Biosynthesis of silver nanoparticles mediated by culture filtrate of lactic acid bacteria, characterization and antifungal activity

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Abstract

Silver nanoparticles (AgNPs) are nanomaterials obtained by nanotechnology and due to their antimicrobial properties have a major importance in the control of various species of bacteria, fungi and viruses, with applications in medicine, cosmetics or food industry. The goal of the paper was to present the results of the research carried out on rapid extracellular biosynthesis of silver nanoparticles mediated by culture filtrate of lactic acid bacteria *Lactobacillus* sp. strain LCM5 and to assess the antimicrobial activity. Analysis of transmission electron microscopy (TEM) micrographs evidenced that the size of AgNPs synthesized using culture filtrates of lactic acid bacteria strain LCM5 ranged between 3 and 35 nm diameter, with an average particle size of 13.84±4.56 nm. AgNPs presented a good dispersion, approximately spherical shape, with parallel stripes certifying crystal structure. Frequency distribution revealed that preponderant dimensions of biosynthesized AgNPs were below 20 nm (94%). Antimicrobial activity of AgNPs was variable depending on both species and group of test microorganisms (bacteria or fungi) involved. Diameter of growth inhibition zone of *Aspergillus flavus* and *Aspergillus ochraceus* caused by silver nanoparticles synthesized by lactic acid bacteria strain LCM5 were similar (12.39 ± 0.61mm and 12.86 ± 0.78 mm) but significant stronger inhibition was registered against *Penicillium expansum* (15.87 ± 1.01mm). The effectiveness of biosynthesized silver nanoparticles was more pronounced against Gram-negative bacteria *Chromobacterium violaceum* with larger zone of inhibition (18 ± 0.69 mm diameter) when compared to those from fungi. Results recommend the silver nanoparticles biosynthesized using culture filtrate of the lactic acid bacteria *Lactobacillus* sp. strain LCM5 for biotechnological purposes, as promising antimicrobial agents.

Keywords: silver nanoparticles, lactic acid bacteria, capping agent, antimicrobial activity, phytopathogenic fungi

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Introduction

Nanotechnologies are involved in finding new inexpensive, rapid and safe solutions for synthesis of nanoparticles, especially silver and gold nanoparticles (1, 2, 3), efficient against spoiling or pathogenic microorganisms producing important economic loss in agriculture and food industry or seriously affecting plants, animals and human health (4, 5, 6, 7, 8).

A lot of research is devoted to biosynthesis of silver nanoparticles (AgNPs) with antimicrobial properties, mediated by various natural sources such as extracts of plant parts (root, stem, leaves, seeds) (9, 10, 11), algae (12,13), bacteria (14, 15) and fungi (16, 17) as an eco-friendly, low-cost and simple alternative to hazardous toxic and expensive chemicals (18, 19). Thus, while physical and chemical synthesis methods use scarce and expensive sources, the biosynthesis of metal nanoparticles can contribute to sustainable development goals (20). Biogenic metal nanoparticles are produced by various microorganisms by either intracellular or extracellular mechanism (21). In the first case, ions are transported into the cell and reduced to their elemental form through electrostatic and enzymatic interaction, with formation of nanoparticles within the microbial cell.

Extracellular mechanism of biosynthesis is mediated either by enzymatic reduction at the cell surface or by secreted molecules that reduce metal ions into their elemental form (22).

Literature reports data concerning with biomolecules, polysaccharides acting as chelating/reducing agents and capping agents for the synthesis of nanoparticles, utilized for preventing aggregation, increasing stability and longevity of the biosynthesized nanoparticles. Considerable efforts have been done to obtain biosynthesized nanoparticles with controlled morphology, small dimensions, uniformity and stability, characteristics favorable to a high antibacterial and antifungal activity (23, 24, 25). Research has been carried out to bring new information for clarifying the mechanisms of antimicrobial action of biosynthesized AgNPs (26), very useful for medicine against clinical pathogens (27, 28).

The goal of the present paper was to present the results of the research carried out on extracellular biosynthesis of silver nanoparticles mediated by culture filtrate of a lactic acid bacteria strain and to assess the antimicrobial activity.

Materials and Methods

Materials and microorganisms:

Lactic acid bacteria strain LCM5 (with origin in brined cucumbers) was cultivated on liquid media MRS broth (purchased from Liofilchem Italy) in test tubes at 36^0C for 24 or 48 hours (29).

The four test microorganisms, represented by mycotoxigenic fungi from species *Aspergillus ochraceus*, *Aspergillus flavus* and *Penicillium expansum* were isolated from contaminated bread, pickles and apples and pathogenic Gram-negative bacteria *Chromobacterium violaceum* was isolated from soil.

Biogenic synthesis of silver nanoparticles

Extracellular synthesis of silver nanoparticles was accomplished by mixing 50ml cell free supernatant from 48 hours liquid culture of lactic acid bacteria strain LCM5 (filtered through membrane filter with 0.2µm pore dimension) with 50ml aqueous solution of 1mM silver nitrate ($AgNO_3$). The mixture was incubated in Erlenmeyer flasks on orbital shaker (200 rpm) at 28±2^0C in the dark for 5 days. A flask with cell free supernatant without $AgNO_3$ was run along with experimental flask and utilized as control (30).

Visual analysis and spectrophotometric characterization of silver nanoparticles

Synthesized nanoparticles color in liquid was visually monitored for changing towards yellowish to brown after adding $AgNO_3$, consequently to completion of reaction, the phenomenon being produced by excitation of Surface Plasmon vibration in silver nanoparticle (31). Aliquots of AgNPs solution (incubated in dark for 24 hours) were taken and optical density (O.D.) was read to a Carl-Zeiss Jena Spectrophotometer at wavelength between 400 and 500 nm against deionized water as blank.

Transmission electron microscopy (TEM) analysis

The size, shape and dispersion of silver nanoparticles synthesized were analyzed by Transmission electron microscopy (TEM) using a JEM – 1400 (Jeol) microscope with an accelerating voltage of 80kV. Three samples were prepared for imaging by drop-coating silver nanoparticles solution on carbon-coated copper TEM grid 40 mm x 40 mm mesh size, air-drying and loading on specimen holder (32). TEM images for each sample were taken at various magnifications and particle size distribution was performed by computer assisted analysis of representative images and grouping the particles counted in categories, according to diameter. The diameter was calculated as average value of two perpendicular diameters for particles assumed as circular or as average of minor and major axes (33).

Antimicrobial activity of silver nanoparticles

Antifungal activity of silver nanoparticles biosynthesized was assessed according to agar well diffusion method (34) against isolates of *Aspergillus ochraceus*, *Aspergillus flavus* and *Penicillium expansum*. Antibacterial activity was assessed against gram negative bacteria *Chromobacterium violaceum.*

30 mL of biosynthesized AgNPs were added in wells of 6 mm diameter in Petri plates with potato-dextrose-agar (PDA) or nutrient agar (NA) media (Merk KGaA, Germany) previously inoculated with test-microorganisms (fungi and respectively bacteria strain). Distilled water and $AgNO_3$ were added in equal quantities in wells as control and plates were incubated at 25°C for 5 to7 days. The diameter of zone of inhibition around the well was measured.

The experiment was performed in triplicate.

Results

Visual analysis and spectrophotometric characterization of silver nanoparticles

After 24 hours incubation of cell free culture with $AgNO_3$ the color gradually changed to dark brown, indicating the production of silver nanoparticles (due to reduction of Ag^+ to Ag^0 mediated by enzyme nitrate reductase) and no color change appeared in control without silver ion Ag^+(**Fig.1**).

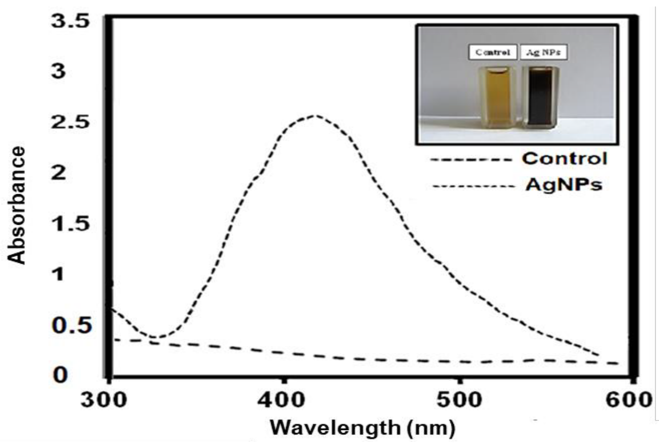

Figure 1. Spectrophotometric analysis of biosynthesized silver nanoparticles. Control - Initial mixture containing *Lactobacillus* supernatant and 1mM silver nitrate in 1:1 ratio. AgNPs - Color change observed of reaction mixture containing *Lactobacillus* supernatant and 1mM silver nitrate in 1:1 ratio

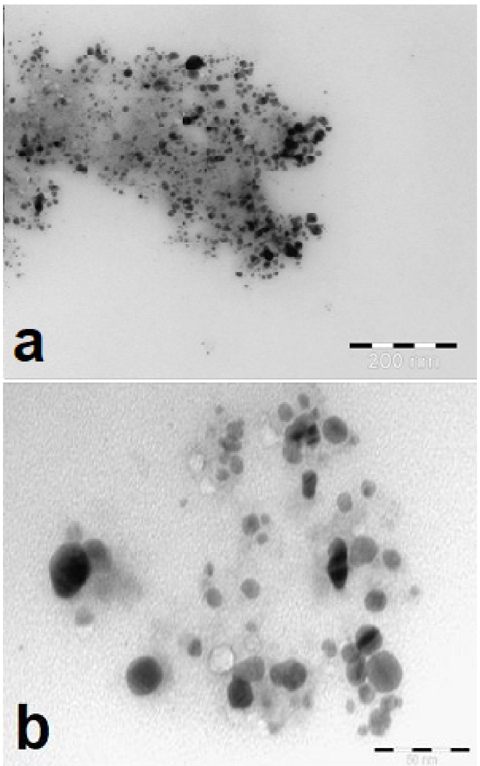

Figure 2. Transmission Electron Microscopy (TEM) micrographs of silver nanoparticles synthesized with culture filtrate of *Lactobacillus* sp. strain LCM5 at different magnifications (a) 30000x, (b) 200000x.

The color of biosynthesized nanoparticles was dark brown due to the excitation of Surface Plasmon vibration in silver nanoparticle. The maximum absorption peak (optical density) occurred at the wavelength of 420nm, as resulted from spectrophotometric analysis.

Transmission electron microscopy (TEM) analysis of AgNPs

Silver nanoparticles synthesized using culture filtrates of lactic acid bacteria *Lactobacillus* sp. strain LCM5 were polydisperse, presented approximately spherical shape, with clear parallel lattice fringes, confirming their crystalline nature. They were embedded in a matrix (acting as capping agent to prevent agglomeration of nanoparticles) as evidenced by transmission electron microscopy (TEM) (**Fig.2**).

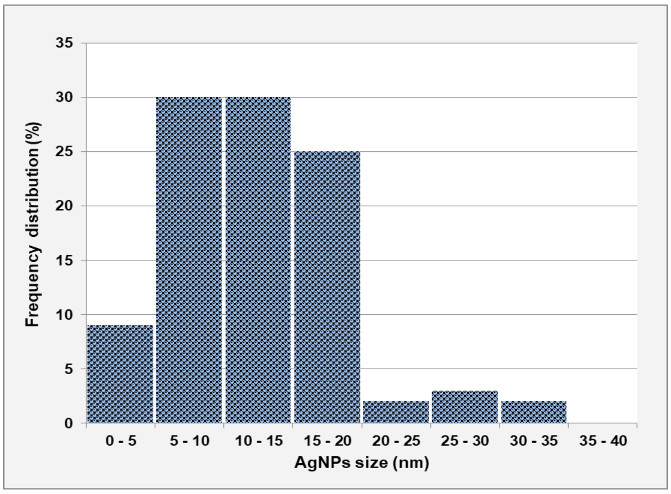

Figure 3. The particles size distribution of synthesized AgNPs.

Analysis of TEM micrographs evidenced that the size of AgNPs synthesized using culture filtrates of lactic acid bacteria *Lactobacillus* sp. strain LCM5 ranged between 3 and 35 nm, with an average particle size of 13.84±4.56 nm.

The particles size distribution of silver nanoparticles showed high values and similar (30%) for 5-10 nm and 10-15 nm categories (**Fig.3**).

Frequency distribution revealed that preponderant dimensions of biosynthesized AgNPs were below 20 nm (94%).

Antimicrobial activity of silver nanoparticles

Silver nanoparticles synthesized with culture filtrate of lactic acid bacteria *Lactobacillus* sp. strain LCM5 exerted an inhibitory effect against all microbial strains tested. The effectiveness (inhibition zone) as provided by agar well diffusion method and presented in **Table 1**, was variable depending on both species and the group of microorganisms (bacteria or fungi) involved.

Diameter of growth inhibition zone of *Aspergillus flavus* (**Fig.4**) and *Aspergillus ochraceus* (**Fig. 5**) caused by silver nanoparticles synthesized by culture filtrate of lactic acid bacteria *Lactobacillus* sp. strain LCM5 were similar (12.39 ± 0.61 mm and 12.86 ± 0.78 mm) but significantly stronger inhibition was registered against *Penicillium expansum*, with inhibition zone diameter of 15.87 ± 1.01 mm (**Fig.6**).

Table 1. Antimicrobial activity of silver nanoparticles

Test microorganism	Inhibition zone (mm) Ag NO_3	Inhibition zone (mm) Ag NPs
Aspergillus flavus	9.00 ± 1.06c*	12.39 ± 0.61c
Aspergillus ochraceus	7.00 ± 0.37d	12.86 ± 0.78c
Penicillium expansum	11.00 ± 0.46b	15.87 ± 1.01b
Chromobacterium violaceum	16.00 ± 0.67a	18.00 ± 0.69a

*Data represent the mean of three replicates ± standard deviation; values in each column followed by the same letter are not significantly different for p<0.05 (Student test)

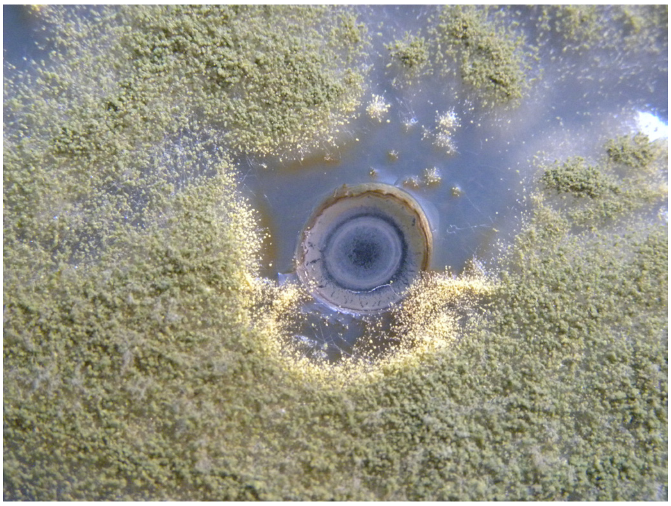

Figure 4. Antifungal effect of AgNPs against *Aspergillus flavus.*

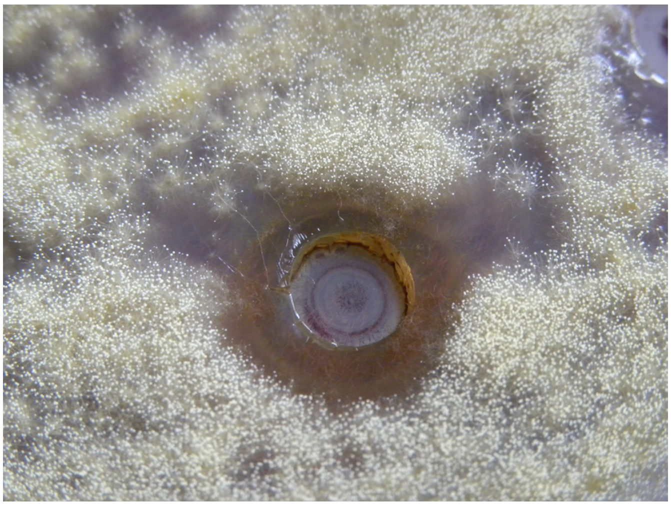

Figure 5. Antifungal effect of AgNPs against *Aspergillus ochraceus.*

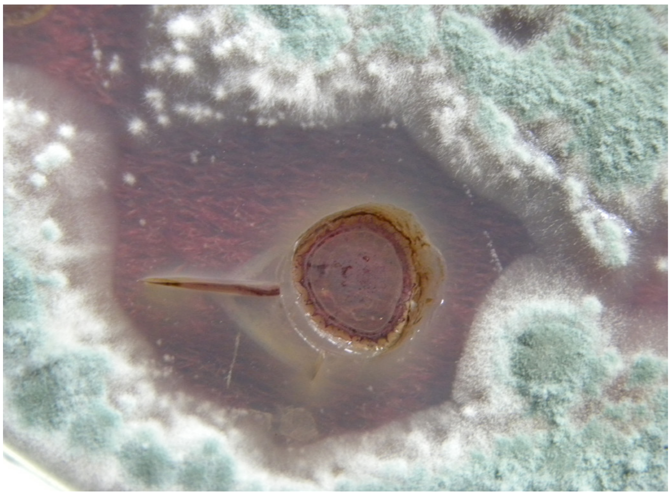

Figure 6. Antifungal effect of AgNPs against *Penicillium expansum.*

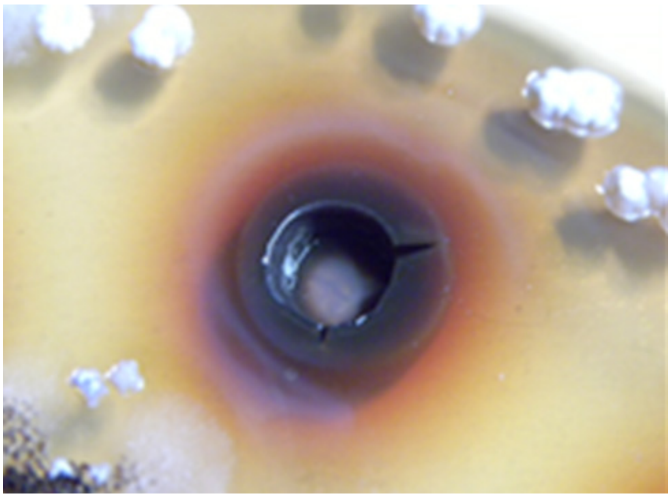

Figure 7. Antibacterial effect of AgNPs against *Chromobacterium violaceum.*

Antibacterial effect of silver nanoparticles against *Chromobacterium violaceum* was evidenced with larger growth inhibition zone diameter (18 ± 0.69 mm) when compared to those from fungi (**Fig.** 7).

Even though both chemical $AgNO_3$ and biogenic AgNPs presented antimicrobial activity against test-microorganisms in the assay, the nanoparticles biosynthesized using culture filtrates of lactic acid bacteria *Lactobacillus* sp. strain LCM5 were more efficient and induced higher values of inhibition zone diameter in the case of bacteria than in the case of fungi.

Discussion

The results of the research carried out demonstrated the extracellular synthesis of silver nanoparticles mediated by culture filtrate of *Lactobacillus* sp. strain LCM5. The color change to dark brown as a consequence of reduction of silver ions with formation of AgNPs and the maximum absorption peak occured at 420 nm weavelength were similar to those occured for AgNPs synthesized by other culture filtrates of lactic acid bacteria from genus *Lactobacillus* (35, 36) or using aqueous leaf extracts (37), with maximum absorption peaks at 430 nm and 437 nm weavelengths.

Biosynthesized AgNPs presented a good dispersion, approximately spherical shape, with parallel stripes certifying crystal structure. The preponderant dimensions of the AgNPs were below 20 nm, with an average particle size of 13.84±4.56 nm. Unlike chemical or physical synthesis, biosynthesis mediated by bacteria culture filtrate presents the advantage to be rapid, eco-friendly (minimal waste generating) and energy efficient as confirmed by similar research (38). Recent highly efficient (92.4% yield) chemical synthesis of AgNPs by one-pot low cost process using monoethanolamine as a strong reducing agent and poly (acrilic acid) as stabilizing agent was reported, with well dispersed, spherical particles of 14.83±5.96 nm and catalytic properties (39). A synthesis of chemical, physical and biological methods for obtaining AgNPs with various shapes and dimensions (from 2 to 300 nm) was presented in an article comparing advantages and disadvantages of these methods and the applications of the nanoparticles in different domains (40).

It is well known that the high frequency of small AgNPs confer the advantage of larger surface of contact with test-microorganisms, increasing their reactivity comparatively with larger AgNPs (41).

Higher intracellular bioavailability of AgNPs associated with increased *in vitro* toxicity for various microbial cells (bacteria, yeasts) was hypothesized to be due to more efficient cell-particle contact of small nanoparticles (10nm) as compared to larger ones (42, 43).

Our results are in concordance with data from research carried out on AgNPs biosynthesized using culture filtrates of lactic acid bacteria strains showing also prominently spherical shape and diameters ranging from 2-20 nm to 20-40 nm, as a function of strain (36, 44). Similar research on lactobacilli found that the ability to reduce the silver ions was strain specific, in most cases the diameter of spherical nanoparticles measured by TEM analysis was between 10 and 40 nm (89.6%) whereas 0.7% were smaller than 10 nm and 9.7% were greater than 40 nm. The monosaccharide composition of capsular heteropolysaccharides from different *Lactobacillus* strains could explain the ability to reduce the silver ions and produce AgNPs with different sizes and size distribution. The differences in antibacterial activity of AgNPs assayed, as in our experiment, by agar well diffusion method were explained by authors as influenced by the capsular biopolymer composition (45). Also, a recent study (46) presented the biosynthesis of AgNPs for biomedical applications, mediated by extracellular pigment produced by *Thalaromyces purpurogenus*, with particles size from 4 to 41 nm diameter, polydispersed and stable, exhibiting a surface plasmon resonance at around 410 nm but a more variable shape of nanoparticles (spherical, hexagonal, rod-shaped or triangular) than in the present research. As in our research, the authors reported a higher number of particles within 10 nm range and a lesser number of larger particles. Green synthesized AgNPs presented higher antibacterial activity against Gram-positive and Gram-negative species assessed by comparing with the effect of Streptomycine.

Similar results with those from our bioassay showing a significant higher antimicrobial activity of biosynthesized AgNPs as compared with $AgNO_3$, were reported by other authors (47) for antifungal activity of biosynthesized AgNPs (assessed by agar well diffusion method, against *Candida* spp.) as compared with $AgNO_3$.

In the present research, the antimicrobial activity of biosynthesized AgNPs maintained over 14 days as illustrated by the aspect of Petri dishes with *Chromobacterium violaceum, Aspergillus flavus*, *Aspergillus ochraceus* and *Penicillium expansum*. The presence of zone of inhibition visible over 7 days was considered as a bactericidal and respectively fungicidal effect, as stated in assays of antimicrobial activity of bacteriocin-producing lactic acid bacteria strains (48).

Recent research evidenced the antibacterial and anti-quorum sensing activity of phytosynthesized silver nanoparticles against violacein producing bacteria *Chromobacterium violaceum* (49) or other human pathogenic species of genera *Escherichia, Staphylococcus, Pseudomonas* and *Bacillus*, with zones of inhibition of 4-18 mm as a function of species and the concentration of AgNPs (50). In the last case, the AgNPs ranged in size from 37 to 43 nm (higher values in diameter as compared with our findings for AgNPs synthesized from bacteria strain) and exhibited varying levels of inhibition of violacein production. Other research (51) with similar results were obtained using agar well diffusion method to assess the antibacterial effect of phytosynthesized AgNPs against various bacteria species causing human urinary tract infections (UTI). The authors reported significant inhibition of all UTI pathogens, with the maximum antibacterial activity of AgNPs synthesized from ginger (zone of inhibition of 18 mm against *Staphylococcus* spp.).

Results from literature reported zones of inhibition from 15 to 20 mm diameter of six fungal species of *Aspergillus* and *Fusarium* caused by antifungal action of AgNPs synthesized using culture filtrates of *Aspergillus foetidus* strain MTCC8876 (52) and powerful antifungal activity of mycosynthesized AgNPs against *Penicillium digitatum*, *Aspergillus flavus* and *Fusarium oxysporum in vitro* (53).

Other research reported AgNPs synthesis mediated by species of genus *Lactobacillus* and their antibacterial action against Gram-positive and Gram-negative human pathogenic species was evidenced by variable growth inhibition zones of 16 to 24 mm in diameter, as a function of species (54). Antifungal action of biogenic AgNPs synthesized using culture filtrates of *Escherichia coli* and *Bacillus subtilis* was suggested to be produced by penetration of AgNPs into the fungal cell and inducing cellular death of clinical isolates of *Candida albicans*, *Trichophyton rubrum* and *Aspergillus fumigatus* (55). Zones of inhibition varying from 8 mm to 14 mm caused by antifungal action of silver nanoparticles biosynthesized using *Allium ampeloprasum* leaf extract were reported for five mycotoxigenic isolates of *Aspergillus* (56).

Recent reviews of antimicrobial activity of AgNPs obtained by green synthesis mediated by various plants and microbes, assessed mainly by agar well diffusion method (57, 58) are consistent with the results from our study concerning the size and shape of AgNPs, and present comparable values reflecting antibacterial and antifungal action.

Results of the present research evidenced the antimicrobial activity of silver nanoparticles biosynthesized using culture filtrate of the bacterial strain LCM5 and recommend it for utilization in biotechnological strategies against pathogenic and food spoilage microorganisms.

Conclusions

Silver nanoparticles biosynthesis was accomplished using culture filtrates of lactic acid bacteria *Lactobacillus* sp. strain LCM5 and aqueous solution of 1 mM $AgNO_3$.

Transmission electron microscopy (TEM) analysis showed a good dispersion of biosynthesized silver nanoparticles, approximately spherical shape, with parallel stripes certifying crystal structure and preponderant dimensions below 20 nm.

Silver nanoparticles biosynthesized using culture filtrate of *Lactobacillus* sp. strain LCM5 presented antifungal activity against *Penicillium expansum*, *Aspergillus flavus*, *Aspergillus ochraceus*, the diameter of zone of inhibition differing depending on fungal species.

Antibacterial activity of biosynthesized silver nanoparticles has been observed against *Chromobacterium violaceum* with more extended zone of inhibition.

Antimicrobial activity of biosynthesized silver nanoparticles was maintained more than 14 days, confirming their bactericidal and fungicidal effect.

Results recommend the silver nanoparticles biosynthesized using culture filtrate of *Lactobacillus* sp. strain LCM5 for biotechnological purposes, as antimicrobial agents.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Ethical Compliance

This article does not contain any studies involving human participants or animals performed by any of the authors.

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