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BIOSYNTHESIS OF SILVER NANOPARTICLES USING CULTURE FILTRATES OF LACTIC ACID BACTERIA AND ANALYSIS OF ANTIFUNGAL ACTIVITY

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The biosynthesis of silver nanoparticles (AgNPs) is a recent approach in nanotechnology with promising application in food industry, pharmacy and medicine. In the present study, the biosynthesis of silver nanoparticles from aqueous solution of $AgNO_3$ was performed using the cell free supernatant of *Lactobacillus plantarum* strain LAB 58. The silver nanoparticles biosynthesis was identified by the change of colour of culture filtrate from yellow to brown due to the excitation of surface Plasmon vibrations by absorbance at 420 nm. Transmission electron microscopy (TEM) analysis revealed that the silver nanoparticles were spherical or polyhedral, with size range from 2 to 45 nm. AgNPs presented antifungal activity against spoilage fungi from genera *Fusarium, Penicillium* and *Aspergillus*. Optic microscopy analysis revealed hyphal alteration of *Fusarium* structures under the influence of treatment with AgNPs. The biosynthesized silver nanoparticles may have application as antifungal agents in food control and medicine.

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Keywords: Silver nanoparticles, TEM, Antifungal activity, Lactic acid bacteria, Cell free supernatant

1. Introduction

Physical and chemical methods utilised for the synthesis of silver nanoparticles proved to be expensive and toxic chemicals can affect the environment and human health. Recent studies showed an increased interest in biological synthesis of silver nanoparticles using microbial (bacterial and fungal), as well as various plant extracts (Table 1).

This method presents the advantage to be time saving and cost-efficient and, the most important, eco-friendly by the absence of toxic compounds during mass production of AgNPs. The antimicrobial activity of silver nanoparticles was proved against various species of bacteria, fungi and viruses. A review of antimicrobial mechanisms involved [13] includes the attachment of nanoparticles of cell membrane, its disruption, penetration inside the cell with negative effects on respiratory chain, DNA and cell death due to oxidative stress induced.

These properties of biosynthesized silver nanoparticles make them promising candidates for using as antimicrobial agents in food safety, pharmaceutical industry and medicine.

The aim of the present study was to synthesize silver nanoparticles by extracellularly reduction of Ag+ ions from aqueous solution of $AgNO_3$ using culture filtrates of lactic acid bacteria isolated from Romanian traditional products (mix of pickle vegetables). The antifungal effect of the AgNPs is reported against four spoilage fungal species.

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Table. 1. Silver nanoparticles of different origins, their size and shape and antimicrobial activity

Synthesizer organism/reducing agent	AgNPs size and shape	Antimicrobial activity (test organisms)	References
Escherichia coli and *Bacillus subtilis*	16-20 nm, spherical, occasionally triangular	*Candida albicans, Trichophyton rubrum, Aspergillus fumigatus*	[1]
Marine *Lactobacillus* sp. (*Lactobacillus plantarum*)	Not analyzed	Multidrug resistant *Candida sp., Fusarium semitectum, Salmonella typhi, Klebsiella pneumoniae, Proteus vulgaris*	[2]
Serratia marcescens S01	30-70 nm, spherical to ovate	*Staphylococcus aureus, Klebsiella pneumoniae*	[3]
Lactobacillus species from VIZYLAC capsules	39-41 nm, prominently spherical	Multidrug resistant hospital isolates *Proteus mirabilis, Salmonella typhi, Klebsiella pneumoniae, Candida sp., Aspergillus niger*	[4]
Lactobacillus sp. VRS-2 from milk	2-20 nm, prominently spherical	Not analyzed	[5]
Lactobacillus species from yoghurt	20-40 nm, different shapes of lone spherical or roughly spherical	*Staphylococcus aureus, Salmonella sp., Shigella sp.*	[6]
Thrichoderma harzianum	30-50 nm, spherical and rod shapes	Not analyzed	[7]
Aspergillus foetidus MTCC 8876	20-40 nm, dominant roughly spherical but also variable in size and shape	*Aspergillus niger, Aspergillus oryzae, Aspergillus parasiticus, Aspergillus flavus, Aspergillus foetidus, Fusarium oxysorum*	[8]
Fusarium oxysorum	50 nm, spherical	Multidrug resistant *Candida sp., Cryptococcus sp.*	[9]
Olive seed extract	10-30 nm, spherical with irregular contours	*Aspergillus niger, Aspergillus flavus, Rhizoctonia bataticola, Alternaria macrospora*	[10]
Fruits waste material	1-10 nm, uniform spherical	*Salmonella sp., Proteus sp., Klebsiella sp., Candida sp., Aspergillus sp.*	[11]
Apium graveolens leaf extract	Closely 25 nm, almost spherical	*Aspergillus niger, Aspergillus wentii*	[12]

2. Materials and Methods

2.1. Materials

Silver nitrate ($AgNO_3$) and PDA utilized for this work were purchased from Merck KGaA Germany. The water used was distilled. The strain LAB 58 was kindly provided by dr. Medana Zamfir (Institute for Biology Bucharest). It was isolated from Romanian traditional products

(mixed pickle vegetables) and genetically identified as *Lactobacillus plantarum*. Fungal species: *Aspergillus ochraceus* (isolated from tomato roots), *Aspergillus flavus* (isolated from contaminated cucumber pickles), *Fusarium verticillioides* sin. *moniliforme* (isolated from contaminated tangerine) and *Penicillium expansum* (isolated from contaminated apple) were used as test microorganisms for the antifungal activity of silver nanoparticles biosynthesized.

2.2. Synthesis of silver nanoparticles using cell free supernatant from LAB 58

Silver nanoparticles biosynthesis was accomplished [14] using 50 ml aqueous solution of 1mM silver nitrate ($AgNO_3$) treated with 50 ml cell free supernatant from 48 hours cultures of *L. plantarum* strain LAB 58. Erlenmayer flasks with mixture were incubated on an orbital shaker (200 rpm) at 28 ± 2^0C and maintained in the dark for 5 days. The control was run along with the experimental flasks, represented by cell free supernatant nontreated with $AgNO_3$.

2.3. Characterization of silver nanoparticles

The characterization of biosynthesized silver nanoparticles was carried out using different techniques and methods including visual observation of colour change of reaction mixture from yellow to brown consequently to completion of the reaction. The silver nanoparticles synthesized exhibit a yellowish-brown colour in water due to excitation of Surface Plasmon vibration in metal nanoparticles [7]. To find the absorbance peak of nanoparticles, aliquots of reaction mixture were taken after 24 hours incubation in the dark and optical density (O.D.) was taken at different wave length ranging from 400 – 500 nm (Carl Zeiss Jena – spectrophotometer).

2.4. Transmission electron microscopy

The transmission electron microscopy (TEM) analysis of silver nanoparticles biosynthesized using cell free culture supernatant of *L. plantarum* (LAB 58) was prepared by drop-coating silver nanoparticles solution on carbon-coated copper TEM grids 40 μm x 40 μm mesh size. Samples were air dried for 10-15 minutes and kept under vacuum in a desiccator before loading onto the specimen holder. Silver nanoparticles size and shape analysis was performed on a JEOL (JEM 1400) instrument with an accelerating voltage of 80kV.

2.5. Antifungal activity of silver nanoparticles

The antifungal activity of silver nanoparticles biosynthesized using cell free culture supernatants of *L. plantarum* (LAB 58) was assessed by agar well diffusion method [8]. Petri plates were prepared by spreading approximately 10^3 CFU/mL from each test fungi on PDA surface. Agar plates were left for 15 minutes before aseptically dispensing 30μL of biosynthesized silver nanoparticles into agar wells (Ø = 6 mm) bored in the agar plate. Equal quantities of distilled water and $AgNO_3$ were dispensed in agar wells utilised as controls. The plates were incubated at 25^oC for 48 hours and inhibition zone diameter around the wells was recorded.

The aspect of fungal structures of *Fusarium verticillioides* before and after the treatment with silver nanoparticles was examined by optic microscopy with MC5 microscope.

3. Results and Discussion

3.1. Visual observation and Spectrophotometric characterization of silver nanoparticles

The cell free culture filtrate of *L. plantarum* strain LAB 58 exhibited gradual change in colour from yellow towards brown when it was incubated with silver nitrate in the dark (Fig.1). Control without silver ion did not presented any colour change of the culture filtrate. The colour of the culture filtrate was dark-brown after 24 hours incubation, indicating the production of silver nanoparticles (due to reduction of Ag+ to Ag0). This is in concordance with literature [5, 15] that reported that the reduction of Ag+ to Ag0 is mediated by the activity of nitrate reductase, a bacterial enzyme released in the solution that can reduce the silver nitrate to silver nanoparticles through capping agents represented by proteins. Research involving lactic acid bacteria included various strains of *L. plantarum.* Data reported for extracellular synthesis of AgNPs by culture

supernatant of *Bacillus subtilis* revealed that the reductase together with electron shuttling compounds and other peptides/proteins may be considered responsible for the reduction of silver ions with silver nanoparticles formation [14].

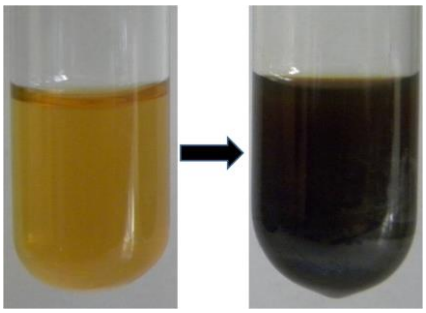

Fig. 1. The colour change of LAB cell free supernatant from light yellow to brown after addition of $AgNO_3$ solution (1 mM) and AgNPs formation

In the present study, nanoparticles synthesis in terms of colour intensity of bacterial cell free culture filtrate was examined at different wave lengths 400-500 nm and absorbance (optical density) was found to be maximal at 420 nm, after 24 hours of incubation.

Similar results were reported for biosynthesis of nanoparticles with marine *Lactobacillus* sp. culture filtrate treated with 1 mM $AgNO_3$, pH 6.0, incubated at 5^{o}C for 24 hours when an absorbance spectra peak registered at 430 nm [2].

3.2. Transmission electron microscopy (TEM) analysis

Transmission electron microscopy provided useful data about the size and shape of silver nanoparticles biosynthesized using cell free culture filtrate of *L. plantarum* strain LAB 58. The high resolution TEM images revealed that silver nanoparticles were polydisperse and predominantly spherical or polyhedral (Fig.2). The images indicate the polycrystalline nature of silver nanoparticles with clear lattice fringes [16, 17].

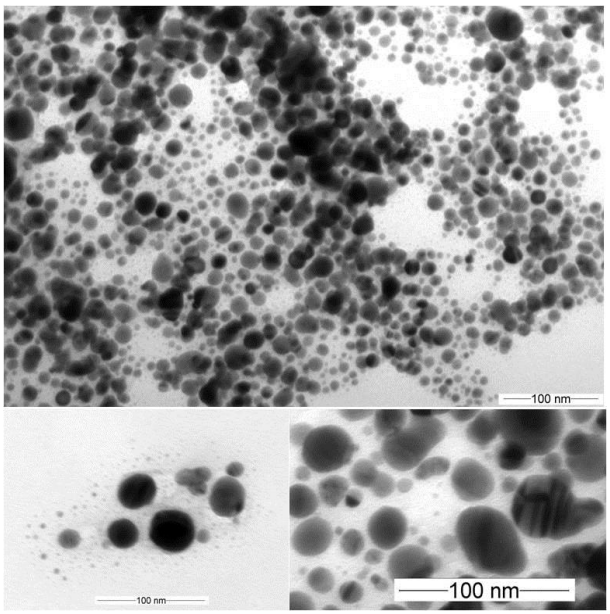

Fig. 2. Transmission Electron Microscopy (TEM) micrographs of synthesized silver nanoparticles

The silver nanoparticles size diameter ranged from 2 to 45 nm. Other research on silver nanoparticles synthesized by *Lactobacillus bulgaricus* reported spherical nanoparticles with size

ranging from 40 to 50 nm [18]. The histogram of particles size distribution (Fig.3) shows that majority is in the range of 15 to 20 nm.

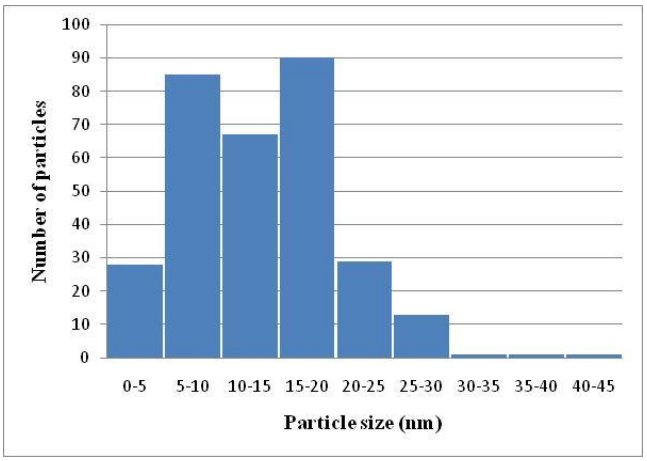

Fig. 3. The particles size distribution of synthesized silver nanoparticles

This is in concordance with the results concerning the nanoparticles size distribution extracellularly synthesized by *Fusarium solani* [19] or by seed exudates of *Sinapis arvensis* [20].

3.3. Antifungal activity of silver nanoparticles

Antifungal activity of silver nanoparticles studied by agar well diffusion method was assayed against fungal isolates of *Aspergillus, Penicillium* and *Fusarium* species responsible for spoilage of fruits, vegetables or other food commodities. Clear inhibition zones formed around agar wells with silver nanoparticles, with higher diameter than those around agar wells filled with $AgNO_3$ and no inhibition zone was observed when distilled water was added into the well (Fig.4).

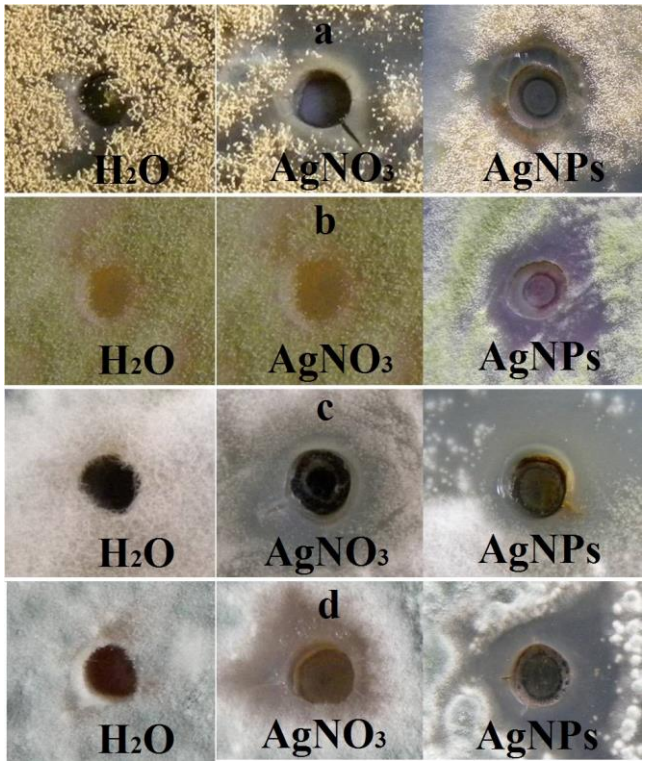

Fig. 4. Antifungal activity of silver nanoparticles (AgNPs) against a) A.ochraceus; b) A.flavus; c) F.verticillioides; d) P.expansum comparatively with silver nitrate ($AgNO_3$) and distilled water (H_2O)

The diameter of inhibition zone (Table 2) varied as a function of the indicator fungal species. The most susceptible fungal species to silver nanoparticles was *F. verticillioides*, where the growth inhibition halo was 18 mm diameter, followed by *P. expansum* and less susceptible *Aspergillus* isolates.

Table. 2. Antifungal activity of silver nanoparticles

Test organism	Inhibition zone (mm) $AgNO_3$	Inhibition zone (mm) AgNPs
A.ochraceus	9 ± 1.06b	10.33 ± 0.47c
A.flavus	7 ± 0.37c	11.66 ± 0.25c
F.verticilliodes	12 ± 0.53a	18 ± 0.82a
P.expansum	11 ± 0.46a	14 ± 0.82b

Data given are 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).

The results are comparable to those obtained for the antifungal activity of silver nanoparticles synthesized by *Aspergillus foetidus* MTCC8876 against six isolates belonging to *Aspergillus* and *Fusarium* species [8]. The authors evidenced by optic microscopy the conidiophores formation was affected when *Aspergillus foetidus* was treated with silver nanoparticles.

The study of fungal structures belonging to *F. verticillioides* by optic microscopy evidenced serious alterations in the inhibition zone created around the agar wells under the influence of silver nanoparticles. Hyphal alteration and delaying in conidial formation are presented in Fig. 5.

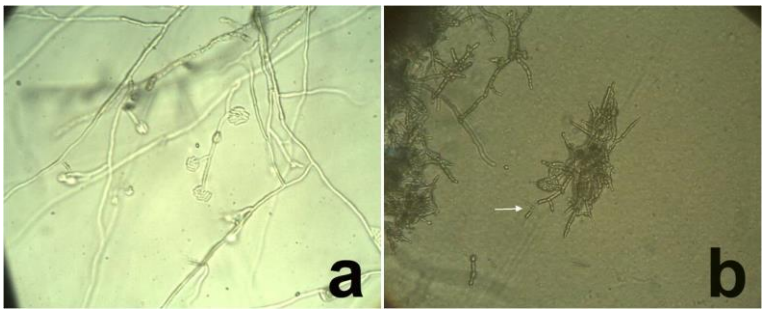

Fig. 5. F.verticillioides micrograph without (a) and with (b) treatment with AgNPs. Arrow indicates hyphal damages with fragmentation points

The mechanism of antifungal activity of silver nanoparticles is not fully elucidated. Results on antifungal activity of AgNPs synthesized by *E.coli* and *B.subtilis* against clinical isolates of *Candida albicans, Tricophyton rubrum* and *Aspergillus fumigates* suggest that the nanoparticles can act by disrupting the structure of the cell membrane, creating of pits, penetrating into the fungi and leading to the cell death [1]. Similarly, the antibacterial activity of AgNPs synthesized using *Acalipha indica* leaf extracts was evidenced by alteration of membrane permeability and respiration of waterborne pathogens *E.coli* and *Vibrio cholerae* [21].

4. Conclusion

The present study demonstrated the possibility to synthesize silver nanoparticles from the aqueous solution of 1mM $AgNO_3$ using cell free culture supernatant of *Lactobacillus plantarum*

strain LAB 58. The size of silver nanoparticles synthesized ranged from 2 to 45 nm and the shape was spherical or polyhedral. The agar well diffusion method proved the antifungal activity of silver nanoparticles against spoilage fungal isolates of *Fusarium*, *Penicillium* and *Aspergillus*. Optic microscopy analysis revealed hyphal alteration of *Fusarium* structures under the influence of treatment with silver nanoparticles. The extracellular synthesis of silver nanoparticles using cell free culture supernatant of lactic acid bacteria is a cost-effective and eco-friendly (non-toxic) alternative method to the expensive and toxic physical and chemical synthesis. The biosynthesized silver nanoparticles may have application as antifungal agents in food control and medicine.

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