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RESEARCH PAPER

Comparative characterization of silver nanoparticles synthesized by spore extract of Bacillus subtilis and Geobacillus stearothermophilus

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ABSTRACT

Objective(s): Silver nanostructures have gathered remarkable attention due to their applications in diverse fields. Researchers have recently demonstrated that bacterial spores are capable of reducing silver ions to elemental silver leading to formation of nanoparticles.

Materials and Methods: In this study, spores of *Bacillus subtilis* and *Geobacillus stearothermophilus* were employed to produce silver nanoparticles (SNPs) from silver nitrate ($AgNO_3$) through a green synthesis method. The production of SNPs by spores, heat inactivated spores (microcapsule) and spore extracts was monitored and compared at wavelengths between 300 to 700 nm. The biosynthesized SNPs by spore extracts were characterized and confirmed by XRD and TEM analyses.

Results: UV-Visible spectroscopy showed that the spore extracts were able to synthesize more SNPs than the other forms. The XRD pattern also revealed that the silver nanometals have crystalline structure with various topologies. The TEM micrographs showed polydispersed nanocrystal with dimensions ranging from 30 to 90 nm and 15 to 50 nm produced by spore extracts of *B. subtilis* and *G. stearothermophilus*, respectively. Moreover, these biologically synthesized nanoparticles exhibited antimicrobial activity against different opportunistic pathogens.

Conclusion: This study suggests the bacterial spore extract as a safe, efficient, cost effective and eco-friendly material for biosynthesis of SNPs.

Key words: *Bacillus subtilis, Geobacillus stearothermophilus, Silver nanoparticles, Spore extract*

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INTRODUCTION

Silver nanoparticles (SNPs) are being used in various fields due to their broad range of application in biotechnology, biomedicine, bioengineering, and electronics [1]. Biosynthesis of SNPs by microbial systems has tremendous advantages over chemical and physical synthesis process. Synthesis route of nanoparticles by microorganisms is biocompatible, environmental friendly, cost effective, and relatively simple [1, 2].

Recently, bacterial spores have been used to produce SNPs through the reduction of silver ions [3-5]. However, there are a few pieces of information about the exact mechanism of nanostructure synthesis by bacterial spores. Different enzymes (glucose oxidase, alkaline phosphatase, laccase, catalase), and carboxylic and hydroxylic groups locating on the surface of endospores have been suggested as the potential factors involved in the biosynthesis of SNPs [3, 6].

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Besides, dipicolinic acid (DPA), which composes up to 15% of the dry weight of endospores, has been proposed to play an important role in the formation of siver nanocrystals [4]. Spores are known to be resistant to toxic compounds, high temperatures, desiccation and different pH ranges [7]. Synthesis of SNPs by bacterial spores is advantageous over other synthesis method due to the stability and availability of spores, simple and rapid route of synthesis and easy recovery of nanoparticles [3-5].

This research was aimed to compare biosynthesis of SNPs by spore-derived components, including whole spores, microcapsules (heat-treated spores) and spore extracts (supernatant of heat-treated spores). Herein, we introduced a simple, rapid, eco-friendly and cost effective biotechnological process for synthesis of SNPs using spore extracts of *Bacillus subtilis* and *Geobacillus stearothermophilus*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Two spore-producing bacteria, *Bacillus subtilis* subsp. *subtilis* (IBRC-M 10997, ATCC 6051) and *Geobacillus stearothermophilus* (IBRC-M 10771, DSM 1550), were employed to produce silver nanoparticles from silver nitrate ($AgNO_3$). These bacteria were cultivated overnight in nutrient broth (Difco) at 37°C, and then inoculated into Difco Sporolation Media (DSM) and cultivated for 24h at 37°C. DSM contained 0.8 % (w/v) nutrient broth, 0.1% KCl, 0.025% $MgSO_4$.$7H_2O$, 1 mM $Ca(NO_3)_2$, 0.01 mM $MnCl_2$ and 0.01 mM $FeSO_4$ in 1 L distillated water, pH 7 [8].

Preparation of spore-derived components

To collect the bacterial spores, the DSM cultures of *B. subtilis* and *G. stearothermophilus* were centrifuged at 13000 *g* for 10 min, and the pellets were used as intact spores to produce SNPs. Then, the spores were heated at 100°C for 10 min to lose their germination ability, and centrifuged at 13000 *g* for 10 min. The pellets, which contained heat inactivated spores, were named "microcapsules" referring to their size and shape. The microcapsules and the spore supernatants (spore extracts) were also collected to be employed as nanoparticle-producing agents [3].

Biosynthesis of silver nanoparticles

An equal volume (1 mL) of spore, spore extract and microcapsule suspensions of *B. subtilis* and *G. stearothermophilus* was added into 50 mL of 1 mM silver nitrate. The reaction mixture without silver nitrate and the silver nitrate solution without the spore components were used as control. The suspensions were incubated at room temperature for 24h to complete the formation of SNPs [3].

UV-Visible spectroscopy

To detect and compare biosynthesis of SNPs by the spores, spore extracts and microcapsules, UV-Visible spectroscopy was performed in the wavelengths between 300-700 nm using UV-160 spectrophotometer device (Shimadzu, Japan).

X-ray diffraction analysis

X-ray diffraction (XRD) analysis of SNPs produced by spore extracts of *B. subtilis* and *G. stearothermophilus* was performed by a Philips X'pert X-ray diffractometer. Data was taken for the 2θ range of 30 to 80 degrees.

Transmission electron microscopy

To study the morphology of SNPs produced by spore extracts of *B. subtilis* and *G. stearothermophilus*, one drop of the sample suspensions was placed on carbon coated copper grids. After one minute, the grids were drained using filter paper, and the silver nanostructures were inspected with a Philips EM 208S transmission electron microscope operating at 100 kV.

Antimicrobial assay

Antimicrobial effects of the SNPs were evaluated on several microbial pathogens such as *Candida albicans*, *Candida glabrata*, *Streptococcus mutans*, *Streptococcus sobrinus*. All strains were multidrug resistant pathogens which had been isolated by our laboratory members. These microbial strains were cultivated in nutrient broth media in the presence different concentrations of SNPs (6, 12, 25, 50 ppm) synthesized by spore extracts of *B. subtilis* and *G. stearothermophilus*. Survival rate of each SNP-treated microorganism was determined by measuring the optical density at 600 nm. The analysis of variance (ANOVA) was used to determine statistically significant differences between the means of three independent replicates.

RESULTS

Biosynthesis of silver nanoparticles

Synthesis of silver nanoparticles was monitored by formation of yellowish brown to dark green colors during 24h incubation of

spores, microcapsules and spore extracts of *B. subtilis* and *G. stearothermophilus* in silver nitrate solution. The silver nitrate solutions containing spores and microcapsules of *B. subtilis* and spores of *G. stearothermophilus* turned to yellowish brown, while, the color of the solutions changed from watery to dark green due to the formation of SNPs by spore extracts of *B. subtilis* and *G. stearothermophilus* (Fig. 1). The production of SNPs by microcapsule of *G. stearothermophilus* was negligible, as shown in Fig 1.

UV-Visible spectroscopy

As shown in Fig 2, the presence of SNPs was detected by UV-Visible spectroscopy absorption spectra ranging from 300 to 700 nm wavelengths with the maximum absorbance between 400 to 500 nm corresponding to the surface plasmon resonance of silver. SNPs produced by spore extracts of *B. subtilis* and *G. stearothermophilus* showed maximum absorbance, and microcapsule-made SNPs represented minimum absorbance at 450nm. Interestingly, the maximum absorbance of both spore extract-synthesized SNPs was significantly more than that of SNPs produced by whole spores (Fig. 2).

As shown, no significant peak was observed in the samples containing SNPs synthesized by microcapsules of *B. subtilis* and *G. stearothermophilus*. Based on the watery color observed in *G. stearothermophilus* microcapsule containing suspension (Fig. 1), it was predictable that the concentration of SNPs in the aqueous suspension is very low, as confirmed by UV-Visible spectroscopy. Furthermore, the maximum absorbance of 1.97 and 1.85 were detected for the nanoparticles produced by spore extract of *G. stearothermophilus* and *B. subtilis*, respectively. Hence, spore extract-synthesized SNPs were further analyzed and characterized by TEM and XRD analyses.

X-ray diffraction analysis

The XRD diffractograms of SNPs produced by spore extracts are shown in Fig 3. Reduction of silver ions to crystalline nanoparticles was confirmed by analysis of XRD peak patterns. As shown, several intense peaks were observed in the 2θ value spectra of both samples relating to different shapes of the silver nanostuctures. As shown, the diffraction patterns were similar to each other, however, the peaks related to SNPs synthesized by spore extract of *G. stearothermophilus* were sharper.

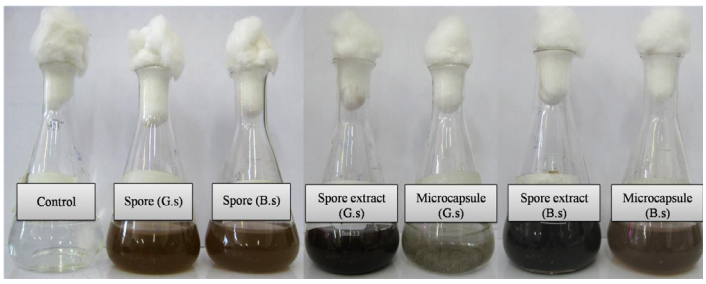

Fig 1. Biosynthesis of silver nanoparticles by spore-derived components of B. subtilis (B.s) and G. stearothermophilus (G.s) in 1 mM silver nitrate solution

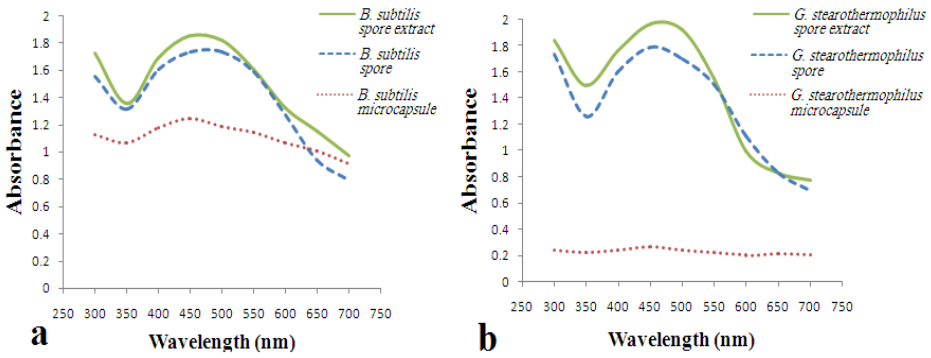

Fig 2. The UV-Visible spectra of silver nanoparticles produced by spore-derived components of B. subtilis (a) and G. stearothermophilus (b)

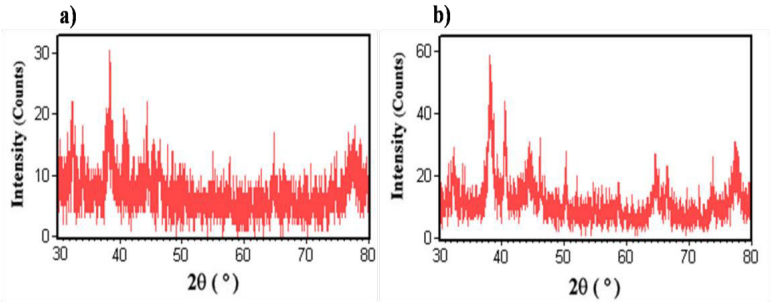

Fig 3. The X-ray diffraction patterns of silver nanoparticles produced by spore extracts of B. subtilis (a) and G. stearothermophilus (b)

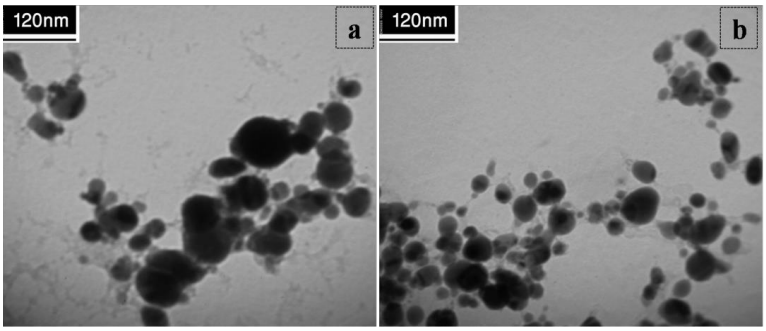

Fig 4. TEM micrographs of silver nanoparticles produced by spore extracts of B. subtilis (a) and G. stearothermophilus (b)

Transmission electron microscopy

TEM analysis was performed to confirm the development of silver nanostructures in silver nitrate solution containing spore extracts, and to compare the shape and size of these nanometals. As shown in Fig. 4, SNPs represented mixed structures with variable sizes ranging from 30 to 90 nm and 15 to 50 nm formed by spore extracts of *B. subtilis* and *G. stearothermophilus*, respectively. Despite of differences in size, TEM analysis of SNPs revealed that the nanostructures are mainly spherical.

Antimicrobial effects of silver nanoparticles

Potential antimicrobial activity of the SNPs was examined on opportunistic pathogens, *S. mutans*, *S. sobrinus*, *C. albicans* and *C. glabrata*. As shown in Fig 5, both spore extract-made SNPs were more effective on the bacterial strains rather than the yeasts. The antiproliferative activity of SNPs was found to be dose-dependent. When the concentration of nanoparticles reduced, a reduction in their cytotoxic effects was observed. Maximum antimicrobial activity was detected after treating the cells with 50 ppm SNPs, as over 97% of bacterial cells and 20% of fungal cells were killed. With some exceptions, the survival rate of bacterial cells treated with SNPs synthesized by *G. stearothermophilus* spore extract was significantly lower than that of the cells treated with SNPs produced by spore extract of *B. subtilis* ($P<0.05$). However, no significant difference was found in antifungal effects of each concentration of SNPs ($P>0.05$).

DISCUSSION

Silver nanoparticles have many applications in diverse fields of biotechnology, including biomedical sciences, diagnostics and biosensor technology.

Due to their antimicrobial properties, SNPs has drawn attention to be widely used in the health, food and textile industries, as well as in a number of environmental applications [9-11].

Up until now, bacterial and fungal biomasses and supernatants, as well as plant extracts were used for green synthesis of nanoparticles [1, 12]. Recently, bacterial spores were also employed to reduce silver ions (Ag^{+}) to elemental silvers (Ag^{0}) leading to formation of nanostructures [3-5]. However, the exact reaction mechanisms of SNPs synthesis by bacterial spores have not yet been clarified.

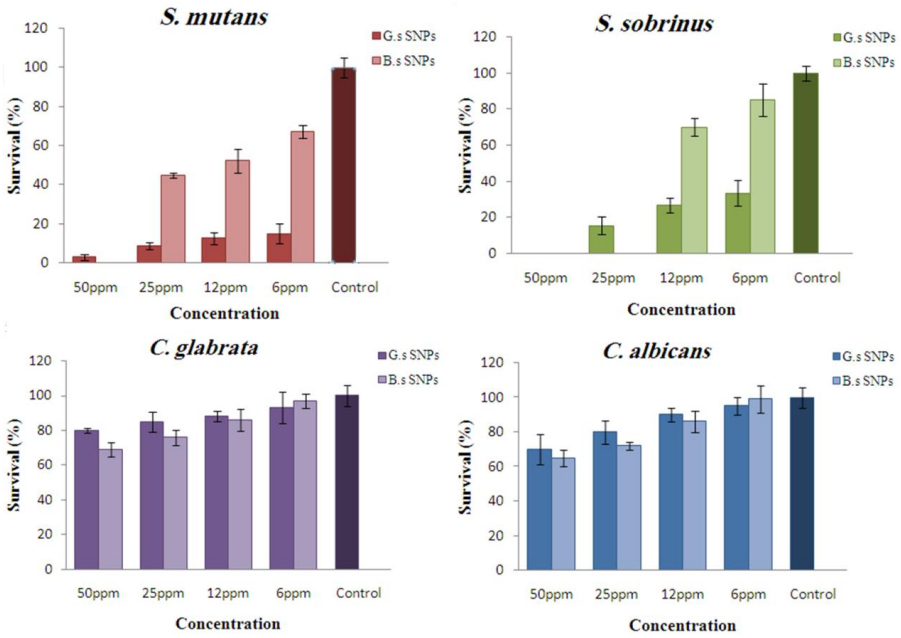

Fig 5. Antimicrobial activity of silver nanoparticles on S. mutans, S. sobrinus, C. albicans and C. glabrata

Different enzymes locating on spores and dipicolinic acid (DPA) locating inside spores were proposed as important factors in reduction of silver ions to SNPs [3, 4]. There are two carboxylic groups in DPA structure. Carboxylic and hydroxylic groups are the other potential factors involved in the biological synthesis of SNPs [6].

In this study, bacterial spore components (intact spores, microcapsules and spore extracts) of *B. subtilis* and *G. stearothermophilus* were used to introduce a cost effective and eco-friendly route for synthesis of SNPs. Each component generated different colors in silver nitrate solution, suggesting different concentration of SNPs (Fig 1). The formation of dark green color in silver nitrate solution inoculated with spore extracts of *B. subtilis* and *G. stearothermophilus* revealed the potential role of DPA in producing high amount of SNPs. The differences observed in UV-visible spectra of spore extract-made SNPs might be related to different concentrations of DPA inside the bacterial spores. Previous studies showed that there is a correlation between color range of the medium and the concentration of SNPs. Accordingly, the color of solution changes from yellow to dark green when SNPs concentration increases from 40 to 200 mg/L [13]. The results of the present study confirmed the correlation between SNPs concentration and color of the medium, as SNPs produced by spore extracts of *B. subtilis* and *G. stearothermophilus* (dark green) showed maximum absorbance and microcapsule-made SNPs (watery to yellowish brown) represented minimum absorbance. According to the results (Fig 1 and Fig 2), the concentrations of SNPs produced by heat-treated spores (microcapsules) of both bacteria were significantly lower than spores- and spore extract-made SNPs. It was probably because of heat inactivation of the enzymes located on the spore surface.

Negligible synthesis of SNPs by microcapsules might be due to the activity of some heat resistant enzymes, especially laccase.

Since spore extracts do not have the germination risk of whole spores, further examination was performed on spore extract-made SNPs to introduce a safe, simple and efficient method for biosynthesis of SNPs. The biosynthesized SNPs were also characterized by XRD and TEM. To our knowledge, the broad spectra observed in UV-Visible spectroscopy, and multiple XRD peaks indicated the biogenesis of polydispersed crystalline nanostructures [5, 14]. TEM micrographs confirmed the development of mixed silver nanostructures with diverse size and typologies (Fig 4). Optical, thermal, magnetic, catalytic and antimicrobial properties of metal nanoparticles depend on their size and shape [15, 16]. According to the results, average size of SNPs produced by spore extract of *G. stearothermophilus* was smaller, suggesting greater antimicrobial potency due to higher surface to volume ratio in comparison with SNPs produced by spore extract of *B. subtilis*.

The action mechanism of SNPs on microbial cells is not completely understood. Accumulation of the nanoparticles on the cell surface, structural changes in the cell membrane, and formation of free radicals by SNPs can be considered as mechanisms by which the microbial cell membranes disrupt, and thereby the organisms die [17- 20]. Furthermore, the silver ions released from nanoparticles can inactivate vital enzymes by interacting with their thiol groups [21].

In this study, the application of SNPs as an antimicrobial agent was investigated on *S. mutans*, *S. sobrinus*, *C. albicans* and *C. glabrata* in nutrient broth media supplemented with silver nanostructures. The results showed that the cytotoxicity of SNPs synthesized by *G. stearothermophilus* spore extract was generally higher than cytotoxic effect of SNPs produced by spore extract of *B. subtilis*, as it was predicted because of their size. In addition, the antibacterial effects of SNPs were significantly more than their antifungal effects. The structural differences between the cell surface of bacteria and fungi may involve in their sensitivity and resistance to SNPs.

CONCLUSION

the present study suggests an efficient, reliable, cost effective and environmental friendly biotechnological process for biosynthesis of silver nanoparticles using the bacterial spore extract. Besides biosynthesis of other nanoscale materials, we propose this promising approach to be used for the bioremediation of silver contaminated environments.

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CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interests.

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