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# Antibacterial Potential of *Bacillus subtilis* Silver Nanoparticles against Some Foodborne Pathogens

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**ABSTRACT**

Silver nanoparticles (AgNPs) are effective antimicrobial agents. This study attends to the extracellular biosynthesis of AgNPs by using *Bacillus subtilis* and its antibacterial activity against some foodborne bacteria. *Bacillus subtilis* produced AgNPs within 72 hrs at 37$^{o}$C in dark conditions while the production occurred through just minutes in the presence of solar irradiation. These nanoparticles were characterized by UV-Vis spectrophotometer showing maximum absorbance at 434 nm. Disk agar diffusion method and micro-dilution assay demonstrate that the produced nanoparticles are potent inhibitors of *B. cereus*, *Staphylococcus aureus*, *Enterobacter cloacae*, *Escherichia coli* and *Pseudomonas aeruginosa*. Thus, the obtained results reveal that these antimicrobial nanoparticles could be used in food industrials as food preservatives.

**Keywords:** *Bacillus subtilis*, silver, nanoparticles, antimicrobial, foodborne bacteria.

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## INTRODUCTION

Bionanotechnology is a new branch of nanotechnology and biotechnology combined for enhancing environmentally benign technology for the synthesis of nanomaterials with specific functions. The diverse application of these materials as catalysts, sensors, food industry and in medicine depends critically on the size and composition of the nanomaterials [1,2]. The chemical and physical methods of synthesis lead to the presence of toxic chemical species adsorbed on their surface which may have adverse effects in the industrial applications [3,4]. The use of toxic chemicals on the surface of nanoparticle and non-polar solvents in the synthesis procedure limits their applications in clinical fields [5]. Hence, the development of clean biocompatible, non-toxic and environmental-friendly methods for nanoparticles synthesis deserves merit. Since biological methods are regarded as safe, cost-effective, sustainable and environment-friendly processes [6]. Researchers have focused their attention on biological systems in the past several years for the low-cost, energy-efficient, and non-toxic production of metallic nanoparticles [4,7]. Microorganisms such as bacteria, actinomycetes, and fungi are investigated in metal nanoparticles synthesis [3,8,9]. Green synthesis of nanoparticles has thus contributed to the development of a relatively new and largely unexplored area of research based on the use of microbes in the biosynthesis of nanomaterials[1,10]. Extracellular production of metal nanoparticles has more commercial applications in various fields. Since polydispersity is a major concern, it is important to optimize the conditions for monodispersity in a biological process [11]. In the case of intracellular production, the accumulated particles are of a particular dimension and with less polydispersity [12]. Among the milieu of natural resources, prokaryotic bacteria have been most extensively researched for the synthesis of metallic nanoparticles such as silver nanoparticles (AgNPs) [7]. Earlier several studies have reported the production of AgNPs by *Bacillus subtilis* 168 [1], *Bacillus* sp. [13] and *B. licheniformis* [14]. The emergence and increase of microbial organisms resistant to multiple antibiotics, and the continuing emphasis on health-care costs, many researchers have tried to develop new, effective antimicrobial reagents free of resistance and cost.

Scientists and industry stakeholders have already identified potential uses of nanotechnology in virtually every segment of the food industry, from agriculture (e.g., pesticide, fertilizer or vaccine delivery; animal and plant pathogen detection; and targeted genetic engineering) to food processing (e.g., encapsulation of flavor or odor enhancers; food textural or quality improvement; new gelation or viscosifying agents) to food packaging (e.g., pathogen, gas sensors ,UV-protection, and stronger, more impermeable polymer films) to nutrient supplements (e.g., nutraceuticals with higher stability and bioavailability) [9,15].

This study aimed to introduce a simple, rapid and cheap method for the biosynthesis of AgNPs, besides, to test their activity against some Foodborne pathogens.

## MATERIALS AND METHODS

### Materials

### Culture media and chemicals

Nutrient agar (NA) medium (Oxoid, UK), Nutrient broth (NB) medium (peptone, yeast extract, NaCl, distilled water) and silver nitrate (PRSpanreac, Barcelona, Spain).

### Microbial cultures

*B. subtilis*, *B. cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter cloacae*, and *Pseudomonas aeruginosa* were kindly performed by Prof. Mohamed I. Abou-Dobara, Professor of Bacteriology, Botany and Microbiology Department, Faculty of Science, Damietta University.

### Methods

### Preparation of bacterial supernatant

*Bacillus subtilis* slants were re-cultured on NA plates. After the incubation period, the bacterial growth was inoculated into 100 mL of NB medium and incubated at 37°C at 150 rpm for 48 hrs to obtain the bacterial

supernatant, the bacterial growth was centrifugated at 5000 rpm for 20 min then the supernatant was transferred into a clean sterile flask.

**Biosynthesis of AgNPs**

The biosynthesis was done by adding $AgNO_3$ (1mM) into the bacterial supernatant (1:1 (v/v %)). The reaction mixtures were done in the presence of the sun irradiation in addition to dark conditions at 150 rpm and 37°C for 72 hrs.

**Characterization of the biosynthesized AgNPs**

The production of AgNPs was characterized firstly by scanning the UV-Vis spectrum (Chemistry department, Faculty of Science, Damietta University). The size and morphology of the produced AgNPs were analyzed using transmission electron microscopy (TEM) (Transmission Electron Microscope JEOL JEM-2100, Japan, Mansoura University, Electron microscope Unit, Egypt). We also determined the surface charge of the synthesized AgNPs by zeta potential analysis using a zeta potential analyzer (Zeta Potential Analyzer, model Malvern Zeta-size Nano-zs90, U.S.A., Mansoura University, Electron microscope Unit, Egypt)

**Antibacterial activity of the biosynthesized AgNPs**

The pathogenic bacterial strains were inoculated into sterile cold melted NA medium and then poured into in sterilized Petri-dishes. The agar plates were left to solidify then holes was made using sterilized cork-borer. AgNPs solutions (100 µl) were added into the holes then incubated at 37°C for 48 hrs. After the incubation period, the zones of inhibition (ZOI) were measured in centimeters.

**Statistical analyses**

Results were expressed as means ± SD. Data were analyzed with one-way Analysis of Variance (ANOVA) for the comparison among bacterial groups, followed by post-hoc Tukey-Kramer multiple comparison tests. The significant level was set at $p < 0.001$ using SPSS version 17.

## RESULTS

**Characterization of the biosynthesized AgNPs**

In the presence of sunlight, the extracellular biosynthesis of AgNPs using *B. subtilis* occurred throughout 1 min due to the color change of the reaction mixture into brown (Fig.1).

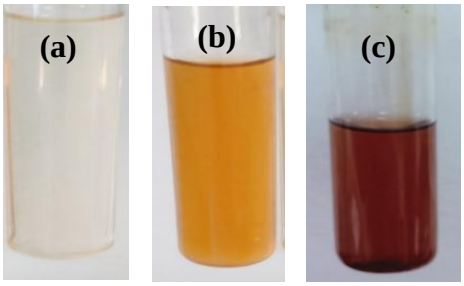


**Fig. 1(a)The dark incubated sample; negative result, (b) Samples after exposure to sun light for 1 min; positive result, (c) The produced AgNPs in the presence of sun light after 111 days.**

The biosynthesized AgNPs produced an absorption peak at 433 nm (Fig. 2). On the other hand, there were no changes observed for the dark incubated samples. The produced AgNPs were analyzed spectrophotometrically after 111 days and give a peak at 456 nm as shown in Fig. 3.

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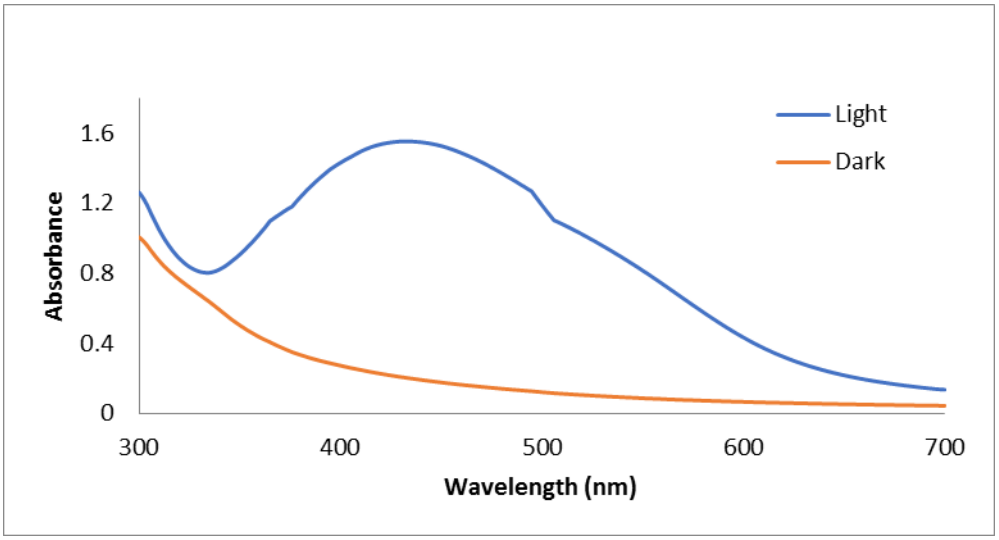


**Fig. 2 UV-Vis spectra analysis**

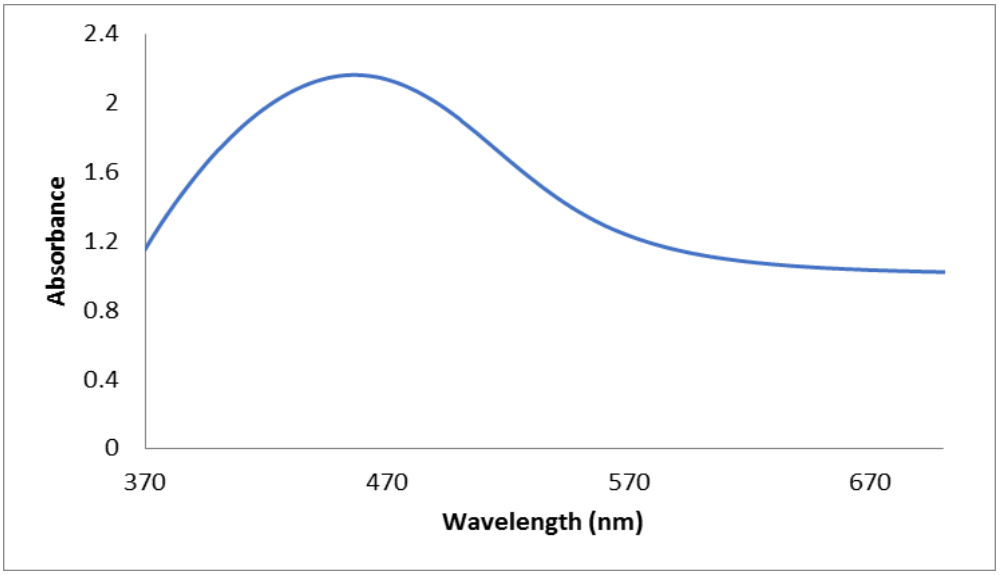


**Fig. 3 UV-Vis spectra analysisof the produced AgNPs in the presence of sunlight after 111 days.**

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Purified AgNPs from extra-cellular culture supernatant were characterized by TEM as shown in Fig. 4. TEM revealed the average size of spherical shaped particles was8-33 nm. In our experiment, we found that the zeta potential of the AgNPs was (-33.5) as shown in fig. 5.

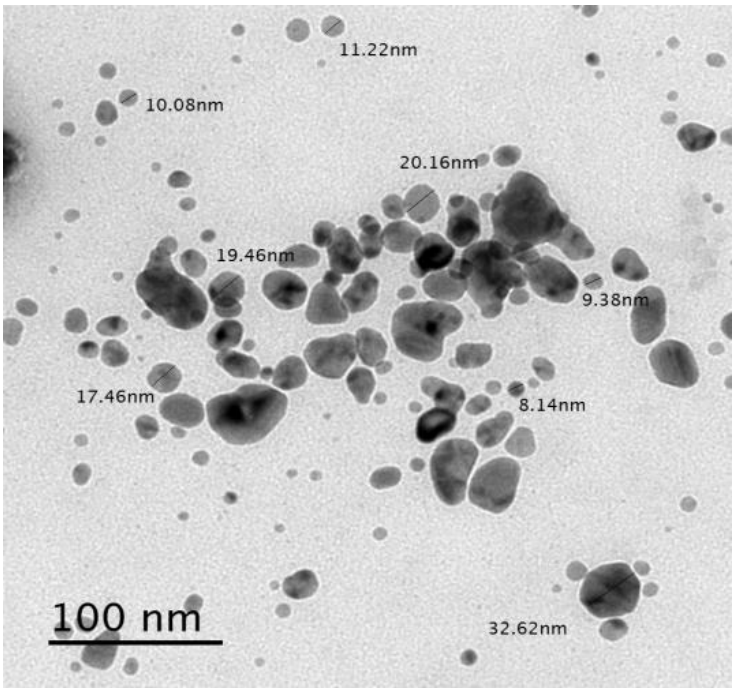


**Fig. 4 TEM micrograph of the biosynthesized AgNPs**

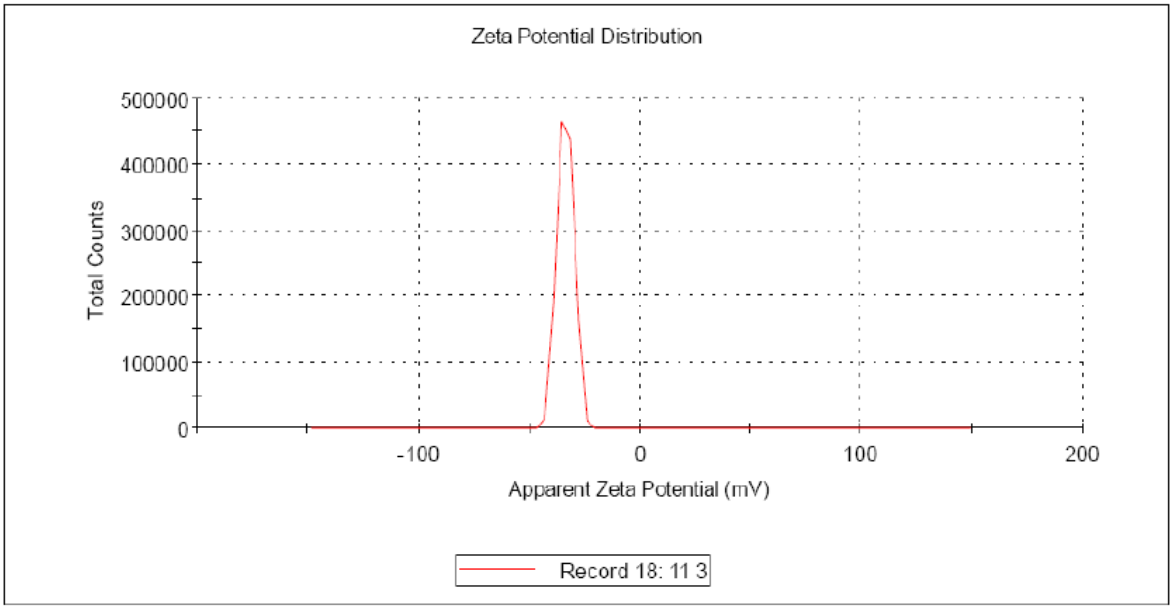


**Fig. 5 The zeta potential of the biosynthesized AgNPs**

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**Antibacterial activity of the biosynthesized AgNPs**

The produced AgNPs showed good potential against the tested Foodborne pathogens as shown in Fig. 6 and Fig. 7.

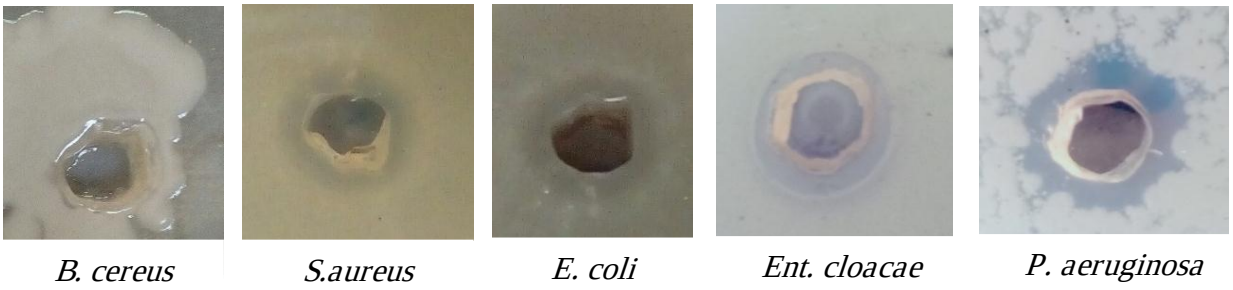


**Fig. 6The antibacterial activity of the biosynthesized AgNPs**

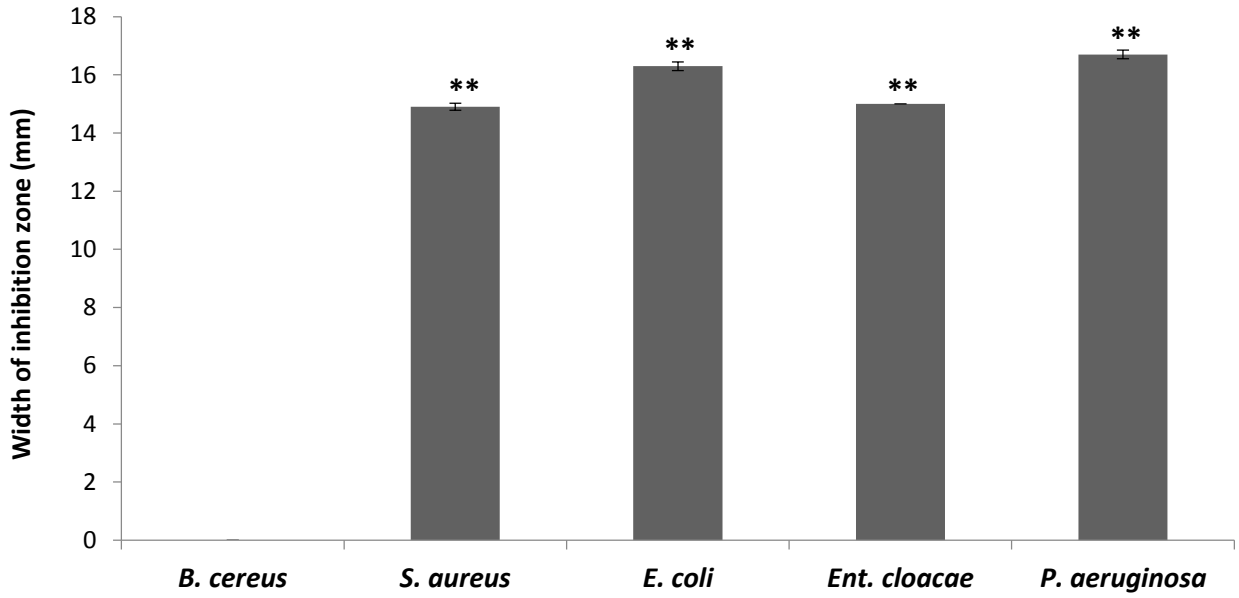


**Fig. 7 The Antibacterial activity (as inhibition zone diameter mm ± SD) induced by the produced AgNPs against the pathogenic bacterial strains. Highly significant = **p<0.001**

Regarding to the statistical analysis, highly significant positive correlations were reported between the bacterial strains *(E. coli, S. aureus, E. coli, Enterobacter cloacae* and *P. aeruginosa*) and the diameter of inhibition zone (r= 0.746, *p*< 0.001). Also, one way ANOVA revealed highly significant differences (*p*< 0.001) between the bacterial species.

## DISCUSSION

Food-borne diseases are of major concern worldwide. Around 250 different food-borne diseases have been described, and bacteria are the causative agents of two-thirds of food-borne disease outbreaks [5]. Among the predominant bacteria involved in these diseases, *S. aureus* which is a leading cause of gastroenteritis resulting from the consumption of contaminated food. Staphylococcal food poisoning is due to the absorption of staphylococcal enterotoxins performed in the food. *S. aureus* is an important pathogen due to a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance [16].

The second pathogen that AgNPs effect on is *Pseudomonas* sp. which may cause spoilage of dairy products due to the production of heat-stable extracellular enzymes. During the pasteurization and UHT treatments, many of these enzymes can survive [17].

The third pathogen that AgNPs effect on is *Enterobacter* spp. *E. cloacae and E. aerogenes* are the most common members of the *Enterobacter* genus to cause infectious disease in humans. Foods such as raw vegetables, dairy products, and raw shellfish are known to harbor these microbes causing foodborne disease [18].

In addition, to being an important member of the normal intestinal microflora of humans and other mammals, the species *E. coli* contains many pathotypes that cause a variety of diseases. At least six different pathotypes cause enteric diseases, such as diarrhea or dysentery, and other pathotypes cause extra-intestinal infections, including urinary tract infections and meningitis. *E. coli* is a predominantly facultative anaerobic Gram-negative bacterium that colonizes the intestinal tract of human infants immediately after birth and helps to maintain normal intestinal homeostasis [19].

In our experiment, the production of AgNPs was observed throughout the colour change into brown colour. The colour changes are due to the surface plasmon resonance of AgNPs in the visible region [1]. The shape, size and potential are important characteristics for the use of nanoparticles in pharmaceutical and industrial applications [15]. The produced AgNPs were spherical in shapeas reported by El-Batal *et al.* [13]. Their sizes were ranged from 8 to 33 nm which matched with El-Bendary *et al.* [20].

The produced AgNPs were charged with negative charges (-33.5), so they can influence bacterial activity as AgNPs can anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane like the permeability of the cell membrane and death of the cell. There is the formation of 'pits' on the cell surface, and there is an accumulation of the nanoparticles on the cell surface [21]. The formation of free radicals by the AgNPs may be considered to be another mechanism by which the cells die. There have been electron spin resonance spectroscopy studies that suggested that there is formation of free radicals by the silver nanoparticles when in contact with the bacteria, and these free radicals have the ability to damage the cell membrane and make it porous which can ultimately lead to cell death [22].

AgNPs have shown to be effective in the reduction of potentially pathogenic bacterial strains, which could reduce the use of antibiotics in livestock. Researchers working in the field of food preservation have reported coating, a simple and versatile technique used for the preparation of coating materials from the colloidal solution containing Ag-NPs using ultrasonication [23,24]. The method proved to be a step forward towards the synthesis of materials with the help of which food can be preserved for a longer duration.

A common problem observed with NPS production is aggregation, which greatly decreases the surface area of the NPS and, in turn, affects their chemical, physical, and biological properties [25,26]. To assess the stability of forming AgNPs in the optimized medium, UV-Vis spectrophotometric study was carried out. In

the case of AgNPs, there was no alteration in the peak at 456 nm even after 3 months of the incubation period. However, the peak for AgNPs got shifted duringafter the third month, but there was no sign of aggregation of NPs comparing with the produced AgNPs by *Bacillus* sp. that were stable for up to 48h as reported by Elbeshehy *et al.* [27].

Future investigations focusing on the mode of action of the produced AgNPs and enable us to find the most efficient AgNPs with higher bactericidal activity and biocompatibility.

## CONCLUSION

*Bacillus subtilis* can biosynthesize AgNPs extracellularly in the presence of solar irradiation. The produced AgNPs are stable and effective antimicrobial agents against some foodborne pathogens especially against *Staphylococcus aureus*, *Enterobacter cloacae*, *Escherichia coli*, and *Pseudomonas aeruginosa*. This study provides a simple and safe method for the production of AgNPs that these antimicrobial nanoparticles could be used as food preservatives.

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