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BIOSYNTHESIS, CHARACTERIZATION AND APPLICATION OF SILVER NANOPARTICLES BY *GEOBACILLUS THERMODENITRIFICANS* AZ1 AS ANTIMICROBIAL, ANTIBIOFILM AND DYE CATALYIST

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Abstract – Silver nanoparticles are playing an important role in biomedical and various applications. Bacteriogenic synthesis of nanoparticles emerges as an eco-friendly and exciting approach. In this work, we demonstrate for the first time the extracellular biosynthesis of AgNPs from the Thermophilic *Geobacillus thermodenitrificans.* The plasmon surface resonance of biosynthesized Ag-NPs showed its characteristic peak at 410 nm. Fourier transform infrared spectrometer (FTIR) analysis confirmed the nature of the capping agents as protein. X-ray diffraction (XRD) and TEM analysis confirmed the purity, spherical and size (1.44 – 16.3 nm) of Ag-NPs. Energy-dispersive X-ray spectroscopy (EDX) data validated the biological synthesis of Ag-NPs. The nanoparticles showed highest antimicrobial activity against *Candida albicans* and to a lesser extent towards *Staphylococcus aureus and Klebsiella pneumonia*. The possible use of NPs (0.15mg/mL) for biofilm eradication was also documented against *S.aureus and P.aeruginosa* (80–90 % inhibition, respectively). In presence of solar energy, 64% degradation of methyl orange (MO) was achieved upon treatment with *G. thermodenitrificans* AZ1 AgNPs.

INTRODUCTION

The biogenic synthesis of silver nanoparticles (AgNPs) has received considerable attention in the last decades due to their unique chemical and physical properties andtheir contribution in different fields including healthcare, food packaging and environment (Calderón-Jiménez *et al.,* 2017). Bacteriogenic synthesis of silver nanoparticles (AgNPs) is more convenient than other ways because bacteria are fast growers, easy to cultivate and handle and able to adapt under extreme conditions (Tripathi *et al*., 2017). Numerous reports are available regarding extracellular synthesis of AgNPs by *Bacillus* species (Saravanan *et al*., 2012; Sunkar and Nachiyar, 2012), Elbeshehy *et al.* (2015) identified new strains of *Bacillus* (*B. pumilus, B. persicus,* and *B. licheniformis*) that were efficient in the AgNPs extracellular synthesis.

Extremophiles, microorganisms living under harsh environmental conditions are one group of microorganisms being utilized for the synthesis of inorganic nanoparticles. Unique living conditions have endowed them with various processes that enable NP biosynthesis. Only few reports are available on the synthesis of silver nanoparticles form thermophilic bacteria. Fayaz *et al.* (2011) reported the synthesis of silver and gold using thermophilic bacterium *Geobacillus stearothermophilus*. The thermophilic *Bacillus* sp. AZ1 was reported to biosynthesize AgNPs extra cellularly (Deljou and Goudarzi, 2016). The Thermophilic *Ureibacillus thermosphaericus* synthesized AgNPs (Juibari *et al*., 2011).

The antimicrobial potential of AgNPs is well-documented in scientific literature as well as in traditional medicine (Zhang *et al.,* 2010) which is attributed to the release of biologically active silver ions upon ionization of silver in aqueous solution (Rai *et al.,* 2009). In addition, the use of $AgNO_3$ as

nano functionalization' surface techniques prevent the formation of life-threatening biofilms on medical devices (Ramachandran and Sangeetha, 2017).

Therefore, in this article we present the first record on the use of the Thermophilic *Geobacillus thermodenitrificans* AZ1 as a nano factory for AgNPs formation. Optimization of the process and characterization of NPs produced are described. In addition, the evaluation of their inhibitory action against some pathogenic microorganisms, biofilm formation and catalytic degradation against some dyes is described.

MATERIALS AND METHODS

Microorganism and cultural condition

A thermophilic *Geobacillus* sp AZ1 applied in previous studies (Abdel-Fattah *et al*., 2012) was investigated here. Itwas maintained on slopes containing Luria-Bertani agar Medium (LBM) (Anthony *et al*., 2013) of the following composition (g/L dist. water): tryptone, 10; yeast extract, 5; NaCl, 10. The strain was stored as spore suspension in 20% (v/v) glycerol at -20 °C.

Bioynthesis of AgNPs

Geobacillus thermodenitrificans AZ1 was freshly inoculated in an Erlenmeyer flask containing 50 mL of LB. The inoculated flask was incubated for 48h in a rotator incubator shaker at 55 °C and 200 rpm. At the end of incubation, the culture was centrifuged at 10,000 rpm for 10 min and the supernatant was used for nanoparticles formation. The biosynthesis of $AgNO_3$ was carried in 100 mL flask containing 10 mL of the suparnatant derived from the bacterial culture, mixed with 10 mL of 1mM $AgNO_3$ and incubated in an orbital shaker at 55 °C, under agitation at 200 rpm in the dark for 48h. Control flasks containing sterile media mixed with 1mM silver nitrate to establish that media components cannot reduce the silver ions to $AgNO_3$ and a negative control ($AgNO_3$ solution) to confirm that no color change is observed by time (Anthony *et al*., 2013). The reaction was monitored visually, as the color of the reaction mixture progressively changed from yellowish to brown, and by a UV-VIS spectrophotometer in range of 200-800 nm (Karthik *et al*., 2014).

Characterization of silver nanoparticles

The silver NPs were subjected to optical absorbance measurement using a UV-Vis spectrophotometer **(**Labomed UV.2800 Inc., Culver City, CA, USA scanning between 200 and 800nm. The presences of nano-silver particles was confirmed by Energy Dispersive X-ray Spectroscopy (EDX) at 20keV. Transmission electron microscope (TEM) was employed to study the size, distribution and morphology of the nanoparticles. XRD analysis of AgNPs was carried in the transmission mode on Shimadzu XRD7000 instrument operating at 40KV current 30mA with Cu Ka radiation (λ=1.5404 A)**.** A monochromatic X-ray beam with wave length lambda was used to analyze the crystalline nature of the sample. The washed pellets of the AgNPs were freeze dried and then analyzed in a FTIR (Perkin-Elmer Spectrum RX1, Shelton,Connecticut device) obtaining the spectrum in the range of 500-4000 cm-1 at a resolution of 1 cm^{-1}.

Biotechnological application of silver nanoparticles

Antimicrobial activity

The agar well diffusion method (Samundeeswari *et al*., 2012) was carried out to determine the antimicrobial activity of the synthesized silver nanoparticles against some human pathogenic microorganism's viz., *Escherichia coli* ATCC 10231, *Klebsiella pneumonia* ATCC 13883, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231.

Anti-biofilm test using tissue culture plate method

P. aeruginosa and *S. aureus* were used as biofilm forming bacteria. The anti-biofilm of AgNPswas performed according to Jo *et al*., (2016). Two concentrations (0.15 and 0.3 mg/mL) of NPs were examined in sterile 96-well flat bottom polystyrene microtiter plate wells inoculated with 100 mL of 108 CFU/mL in TSB and loaded with NPs. For comparison, metals ions, antibiotics and chlorine solutions were also used. The plates were incubated in static condition at 37 °C for 24h. Positive control wells were maintained with medium containing only bacterial suspension, while negative control wells contained sterile TSB only. The contents of wells were removed by tapping the plates and washing three times with sterile phosphate buffer saline to remove loosely attached bacteria (planktonic). Wells were stained with 150 mL of 0.25% crystal violet and incubated for 30 min. Further these wells were washed, air dried, bound

stain was solubilized in 150 mL of 95% ethanol and the absorbance at 595 nm was recorded using the plate reader (Tecan Infinite M200, Switzerland). These optical density (OD) values were considered as an index of bacteria adhering to the surface and forming a biofilm. However, the lower absorbance value revealed the more intensive anti-biofilm effect. The percent inhibition of the biofilm activity was calculated as described in the following equation:

% Inhibition of adhesion = [(A- A0/ A) × 100]

Where, A represents the absorbance of the positive control wells and A0 reflects the absorbance of the treated wells with antimicrobial agent (Ibrahem *et al*., 2014).

Phytocatalytic degradation

The photocatalytic degradation of methyl orange was assessed. The whole test method was conveyed in the open-air with sun as the principle origin of light. A colloidal suspension was prepared by mixing 20 mg of AgNPs with 50 mL of 20 mg methyl orange solution and stirred for 30 min in darkto affirm uniformity of solution. The suspension was kept under daylight into a measuring glass at the time of the reaction. Examinations were completed on a sunny day between 11 AM and 4 PM. The absorption spectrum of the suspension was measured intermittently utilizing an UV-visible spectrophotometer after centrifugation to guarantee the degradation of methyl orange solution (Sinha *et al*., 2015). The percentage of dye degradation and its variation with exposure time is calculated according to following formula

Dye degradation % = (C0 –Ct / C0)* 100

Where C_0 represents the initial concentration of methyl orange, C_t is the concentration of methyl orange dye after exposure to solar radiation for t h.

RESULTS AND DISCUSSION

Biosynthesis of AgNPs by Geobacillus thermodenitrificans AZ1

G.thermodenitrificans AZ1 synthesized silver nanoparticles as indicated visually by change of the yellow color reaction mixture to dark brown. No color change was observed in aqueous $AgNO_3$ incubated without cell free supernatant (Fig. 1). The specific surface Plasmon resonance (SPR) spectra of silver nanoparticles produced by the by strain AZ1 revealed an absorption peak at 410 nm

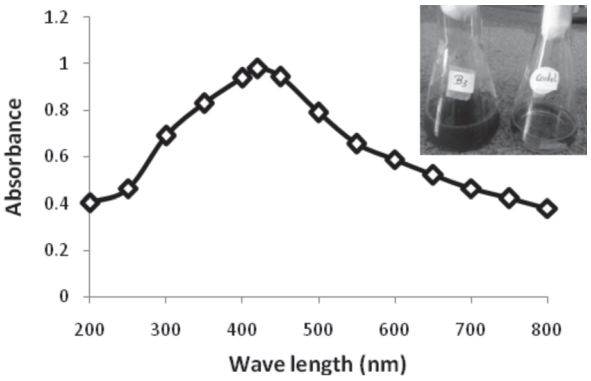

Fig. 1. UV-Vis absorption spectrum of silver nanoparticles synthesized by cell free extract of *G. thermodenitrificans* AZ1.

Characterization of silver nanoparticle

Transmission Electron Microscope (TEM)

Particle size of AgNPs is highly affecting its properties and applications (Kiss *et al*., 2011). TEM micrograph of *G. thermodenitrificans* AZ1 AgNPs showed uniform spherical particles with an average size of 1.44 – 16.3 nm. These are smaller than AgNPs formed by the Thermophilic *G. stearothermophilus* showing size of 5–35 nm (Fayaz *et al*., 2011). They are even smaller than those formed bythe thermophilic *Ureibacillus thermosphaericus* of the size range of 10–100 nm (Juibari *et al*., 2011).

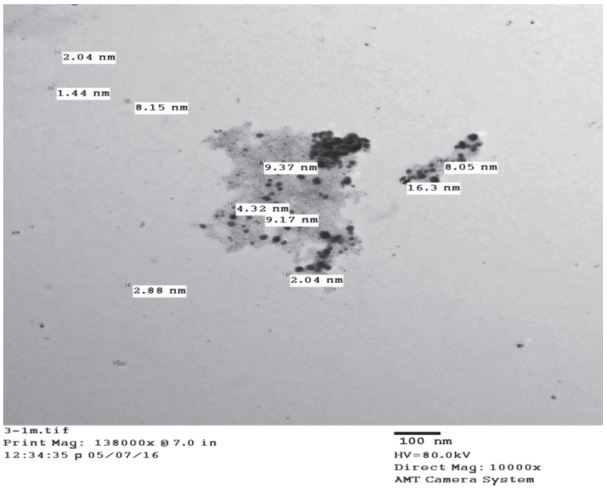

Fig. 2. TEM image of *G. thermodentrificans* AZ1 produced extracellularly.

EDX analysis

EDX is a compositional analysis that gives qualitative as well as quantitative status of the elements that may be involved in formation of

AgNPs (Jyoti *et al.,* 2016). EDX spectroscopy analysis of strain AZ1 AgNPs confirmed the presence of elemental silver (Fig.3). The elemental profile of AgNPs produced showed typical characteristic absorption peak of silver at approximately 3 KeV which is due to the absorption of metallic silver nanocrystallites corresponding to surface plasmonresonance (Singh *et al.,* 2015). Our results agree with those of El-beshehy *et al*., (2015) who reported that the optical absorption peak of silver nano-crystals produced from *Bacillus* spp. arises at about 3 KeV.

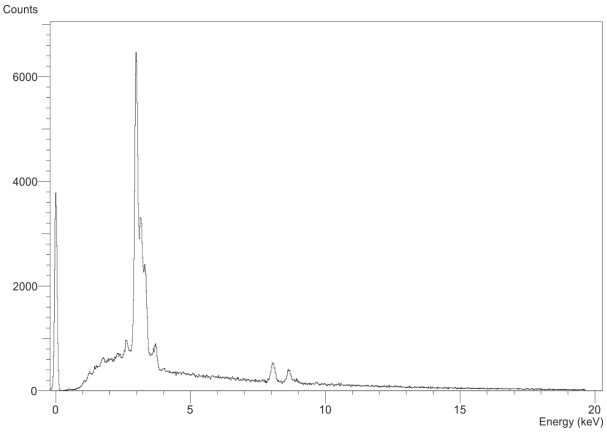

Fig. 3. EDX spectrum of *G. thermodentrificans* AZ1AgNPs showing peak between 3 and 4 KeV.

X-ray diffraction (XRD) analysis

The XRD pattern of nanoparticles exhibited intense peaks in the whole spectrum of the 2θ value ranging from 20 – 80, and this pattern is similar to the Braggs's reflection of silver nanocrystals. AgNPs distinguished XRD peaks with 2' values of distinguished XRD peaks with 2' values of 38.3, 44.44, 64.12, 77.12 and 81.74 were observed (Fig. 4). These peaks are analogous to (111), (200), (220), (311) and (222) reflection planes of face-centered-cubic (fcc) silver, respectively. Our results are on line with those synthesized by *Ureibacillus thermosphaericus* that showed four peaks at 38.1°, 44.5°, 64.6° and 77.62° corresponding to the (111), (200), (220) and (311) of the face-centered cubic silver, respectively (Juibari *et al.,* 2011). Also, Taran *et al*., (2016) mentioned that the X-ray diffraction pattern of the silver nanoparticles synthesized by *Bacillus* sp. showed at 2θ values of 38.09°, 46.09°, 64.52°, 77.51° corresponding to XRD planes (111), (200), (220) and (311) Bragg's reflection based on the fcc structure of AgNPs.

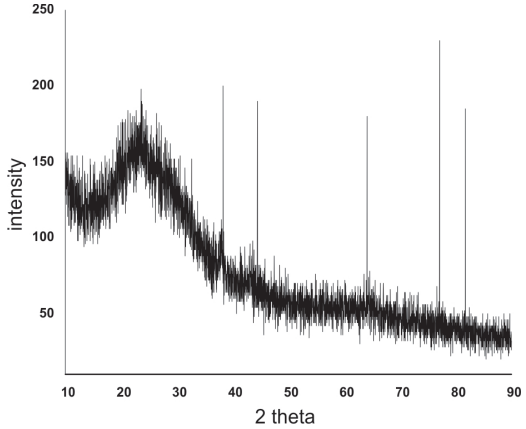

Fig. 4. XRD pattern of AgNPs synthesized by culture supernatant of *G. thermodentrificans* AZ1

Fourier Transform Infrared (FTIR) analysis

FTIR analysis was performed to identify the biological molecules responsible for synthesis and capping of AgNPs (Rani *et al.,* 2017). Fig. 5 shows the FTIR spectrum of AgNPs formed by *G. thermodentrificans* AZ1. The presence of bands at 1045.89 cm^{-1} in the FTIR spectra is attributed to C-O stretching vibration (Chao *et al.,* 2012). The FTIR band at 1385.38 cm^{-1} corresponded to C=C stretching of an aromatic amine group or C-N stretching vibrations of aromatic amines (Faramarzi and Forootanfar, 2011), suggesting that the capping agent of biosynthesized nanoparticles possesses an aromatic amine group. In addition, the presence of bands at 1638.38 and 1763 cm^{-1} in the FTIR spectra is attributed to -C=O stretching vibrations in amide linkages (amide II) of protein present in bacterial supernatant (Hudlikar *et al.,* 2012). The bands seen at 2396.83 and 3409 cm^{-1} is typical of the stretching vibration mode of O–H stretching vibrations (Das *et al.,* 2006). Our results are in agreement with those reported by El-beshehy *et al.,* (2015) that the analysis of silver nanoparticles produced by *Bacillus* spp.

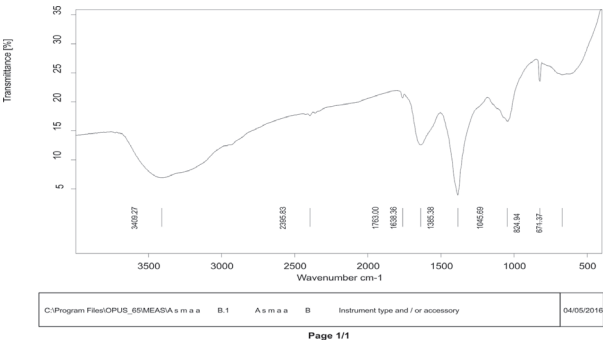

Fig. 5. FTIR spectrum of G. thermodentrificans AZ1 $AgNO_3$

Also, El-Bata *et al*., (2013) reported that FTIR of synthesized AgNPs produced by *Bacillus stearothermophilus* showed bands around 1635.34/cm (correspond to a primary amine NH band), 1388.5/cm and 1118.51/cm (correspond to a secondary amine NH band and primary amine CN stretch vibrations of the proteins, respectively).

Biotechnological application of silver nanoparticles

Antimicrobial activity

The fact that bacterial resistance to elemental silver is extremely rare emphasizes the increased interest in using AgNPs as potent antimicrobial agent in biomedical application (Ottoni *et al*., 2017). Silver nanoparticles have received greater attention as antimicrobial agents (Erjaee *et al.*, 2017) because of the development of antibiotic resistant pathogens. Therefore, the significant antimicrobial activity of AgNPs against many pathogens may pave the way toward formulation of new antimicrobial agents (Patra and Baek, 2017).

As shown in Fig. 6, *Candida albicans* was more sensitive to Ag nanoparticles, followed by Gram-ve *Pseudomonas aeruginosa* then Gram+vebacteria *Staphylococcus aureus*. The different susceptibility of Gram-positive and negative bacteria to AgNPs, is probably due to differences in their membranes and cell walls (Devi and Joshi, 2015). In good agreement with our results are those obtained by Jo *et al*., (2016).

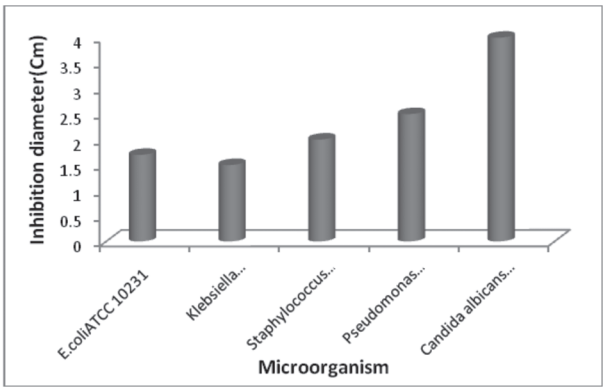

Fig. 6. Antimicrobial activity of *G. thermodenitrificans* AZ1 $AgNO_3$ againstsome pathogens.

Anti-biofilm effect by tissue culture plate method

About ~65–80% infections occurred by biofilm formation microbes, amid which the Gram-negative bacterium *P. aeruginosa, E. coli*, and the Gram-positive *Staphylococci, S. aureus* are the most common ones (Joo and Otto, 2012)**.** Inhibition of biofilm formation and disruption of preformed biofilms can be achieved employing bacterial AgNPs (Gaidhani *et al*., 2013). Exposure to AgNPs leads to reduction in microbial biomass and surviving cells and also inhibition of exopolysaccharide and protein production (Zhang *et al*., 2014).

The data in the present study (Fig. 7) show that at low concentration of AgNPs (0.15 mg/mL) almost 86 and 80% inhibitions were recorded for *S.aureus* and *P.aeruginosa* biofilms, respectively. It is worth mentioning that the anti-biofilm efficacy of NPs was higher than other anti-biofilm compounds tested. The data validate that Ag-NPs can effectively and rapidly detach biofilm, produced by *P. aeruginosa*, and *S. aureus* which implies the application of these Ag-NPs as biofilm-disrupting agents. Goswami *et al*., (2015) also studied the Ag-NPs mediated biofilm eradication, and found inhibition of 89 % for *S. aureus* and 75% for *E. coli* at 15 mg/mL which is much higher than the concentration used in this study.

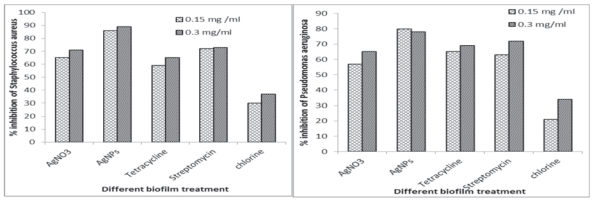

Fig. 7. Inhibition percentages caused by AgNPs of *G. Thermodenitrificans* AZ1 silver nanoparticles on biofilm biomass of *S. aureus* and *P. aeruginosa*

Our results agree with El-tarahony *et al*., (2016) who reported that AgNPs produced by *Achromobacter* sp. caused the highest reduction in the biofilm biomass with 93 and 86 % inhibition at 0.3 mg/mL for *S. aureus* and *P. aeruginosa*, respectively. Chaudhari *et al*., (2012) mentioned that AgNPs produced by *B. cereus* prevent formation of *Staphylococcus aureus* biofilm.

Phytocatalytic degradation of synthesized AgNPs

The degradation of the MO dye in presence of AgNPs was ensured by the steady decrease in absorption peak intensity at 463 nm within 5h of experimental run. On the other hand, the control sample exhibited no variation of color or peak intensity during the time of experiment. It can be inferred from Fig. 8 that 64% degradation of methyl orange was achieved upon treatment with *G.*

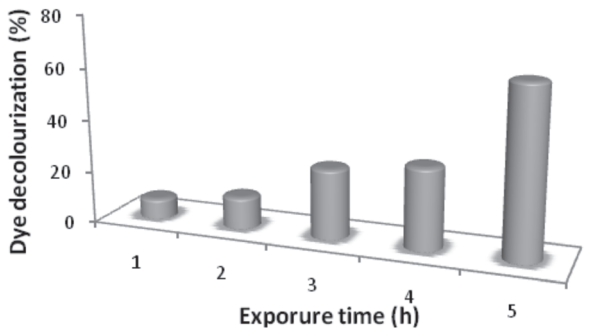

Fig. 8. Percentage of dye degradation versus different exposure time by using AgNPs synthesized by *G. thermodenitrificans* AZ1.

thermodenitrificans AZ1 AgNPs. Percentage of degradation obtained in this study is lower than that found with AgNPs of *Aspergillus wentii* (88%) (Biswas and Mulaba-Bafubiandi, 2016). When compared with other irradiation methods, solar irradiation is more capable in degrading methyl orange in the presence of nanosized metal catalysts (Kavitha *et al*., 2014; Roy *et al*., 2015).

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