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Characterization of Ag nanoparticles biosynthesized by *Bacillus* sp. HAI4 in different conditions and their antibacterial effects

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ABSTRACT

In the present study, Ag nanoparticles (NPs) were synthesized by eco-friendly method. Design and analysis of Taguchi experiments was done by the Qualitek-4 software. Effect of $AgNO_3$ concentration (0.1, 0.01 and 0.001 M), incubation and culturing time (48, 72, 96 hours) as three different levels were measured in NPs biosynthesis. We carried out biosynthesis of NPs through cell-free culture supernatant of *Bacillus* sp. HAI4. X-ray diffraction (XRD), Fourier Transform Infra-Red spectroscopy (FT-IR), Ultraviolet–Visible spectroscopy (UV) and Field Emission Scanning Electron Microscope (FESEM) techniques were applied for NP characterization. Results of Taguchi analysis showed optimum condition with 12.641% amount which is good result of biosynthesis by this bacterium. XRD analysis proved the crystalline and amorphous nature of synthesized silver NPs. Analysis of FESEM demonstrated that Ag nanoparticles were formed as spherical shapes with size of 33/264 nm. Also, for evaluating of antibacterial effects, maximum zone of inhibition, two important multidrug resistant pathogenesis bacteria, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 43300 were used. Three levels of Ag NPs concentrations had antibacterial effect on these bacteria (1, 1 and 0.5 cm for *E.coli* and 1, 1.1 and 1.1cm for *S.aureus*), but there was no significant difference between three levels.

INTRODUCTION

Improving disinfection methods in order to fight against pathogenesis microorganisms specifically multidrug resistant bacteria are important in hospital infections (Varshney *et al.,* 2012). For many years, silver ions were common antibacterial that applied in catheters (Jain and Pradeep, 2005). In this case, with knowledge of bactericidal effect on *E. coli* and *Bacillus subtilis*, copper and silver ions have been used as antibacterial agents in wastewater of hospitals (Lin *et al.,* 1996; Lin *et al.,* 1998; Blanc *et al.,* 2005; Yoon *et al.,* 2007).

These applications of ions have major disadvantages. As, in these wastewater, silver and copper ions can be remained and act as harmful agents for human and environment health (Blanc *et al.,* 2005).

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Nanotechnology and nanoscience are improving these activities of metals nanoparticles (NPs) through unique properties at nanometer scales (10-500 nm, seldom more than 700 nm). These new functions of metal NPs are related to several properties such as surface area to volume ratio which are higher than conventional ion forms of metals (Vicky *et al.,* 2010).

Also, NPs have other applications as drug carriers, catalysis and sensors, etc. (Vaseashta and Dimova-Malinovska, 2005).

Nanoparticles synthesis can be obtained through physicochemical and biological techniques. Chemical synthesis methods have the harmful effect on the environment because using hazardous chemical substances (Monika *et al.,* 2015). In contrast to physicochemical methods, biological syntheses have several advantages such as eco-friendly effects. Bacteria, yeast, algae, fungi, higher angiospermic plants and weeds are used for nanoparticles fabrication (Varshney *et al.,* 2012). Among these biological methods, bacteria have been more considerable because its simplicity and pure production (Vidhya *et al.,* 2014).

Extracellular and intracellular ways can occur in metal NPs synthesis bay bacteria (Kalishwaralal *et al.,* 2010; Saifuddin *et al.,* 2012). Further treatments for release the metal NPs are requiring in intracellular NPs synthesis such as ultrasound and centrifugation (Ganesh Babu and Gunasekaran, 2009). In contrast, simplicity, cheap and large-scale production properties are related to extracellular NPs biosynthesis. Therefore, in experimental studies, extracellular NPs synthesis has been more attention than intracellular one (Vidhya *et al.,* 2014). In this method, Ag NPs can be formed within few minutes from Ag+ ions in the culture supernatant of *Escherichia coli* ATCC 8739, *Bacillus subtillis* ATCC 6633, *Streptococcus thermophiles* ESh1(Abd *et al.,* 2011) and *Streptomyces* sp. ERI-3(Faghri Zonooz and Salouti, 2011).

In this study, selected strain *Bacillus* sp. HAI4. was applied to reduction of Ag^+ ion and conversion it to Ag nanoparticles. UV-visible spectroscopy, X-ray diffraction (XRD), Fourier Transform Infra-Red spectroscopy (FT-IR), and Scanning Electron Microscopy (SEM) techniques were applied for NP characterization. Also, antibacterial effects of the final reaction were measured on two pathogenesis bacteria strain with multidrug resistant property, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 43300.

MATERIAL AND METHODS

Taguchi methodology experimental design

In order to optimization of experimental conditions, all the combination experiments were conducted using the assigned parameter values. The Qualitek-4 software was utilized to design and analysis of Taguchi experiments (Taran *et al.,* 2015). Table 1 demonstrates variable factors and their levels in this experiment design.

Microorganism in Ag NPs biosynthesis and preparation of supernatant

Bacteria *Bacillus* sp. HAI4 was obtained from bacterial archive, Razi University, Kermanshah. Growth conditions were simple, growth on 0.5 NB (nutrient broth) agar plate. The obtained biomass was washed with phosphate buffer (pH 7.0) thrice and collected in a 500-ml Erlenmeyer flask. 1M solution of $AgNO_3$ was prepared using de-ionized water, and 100ml of the solution was added to the biomass harvested at each point of time. The Erlenmeyer flasks were incubated at 37 ˚C under agitation (200 rpm) for 24 h.

Characterization

In order to determination of structure, morphology and elemental composition, the prepared annealed samples were analyzed by X-ray diffraction analysis (XRD), scanning electron micrograph (SEM). Crystallographic study was carried out using EQUNIOX 3000, diffractometer in the scanning range of 20˚ - 70˚ (2θ) using Cu Ka radiations of wavelength 1.5406 Å. Model XL30, Philips, Eindhoven, Field Emission Scanning Electron Microscope (FESEM) was used to study the morphology of the nanoparticles and the elemental analysis. The intensity of absorption peaks and peak absorbance of NPs was examined by UV-Vis spectrophotometer (Tomas, UV 331) from 300 to 800nm. FT-IR measurements were done by spectrophotometer (Germany, Bruker, Model:ALPHA).

Antibacterial effects

Escherichia coli ATCC 25922 and *Staphylococcus aureus* ATCC 45500 as pathogen bacteria were utilized for measurement the effect of antibacterial properties of AgNPs by well diffusion modified ager. Clinical pathogen bacteria were cultivated in Muller Hinton agar (MHA) plates; 5 mm diameter disks were prepared with the help of a sterilized steel cork borer. Afterwards, different concentrations of AgNPs (0.1, 0.01 and 0.001M) were loaded in different disks and after placing the disks on agar the plates were incubated at 37℃ for 48hr (Ramyadevi *et al.,* 2012).

RESULTS and DISCUSSION

The formation of silver nanoparticles was carried out by the culture supernatants of *Bacillus* sp. HAI4. In this case, bacterial strains were incubated for reduction of silver ion to silver NPs as there was rapid reaction (rapid color change at 10 minutes). Figure 1 shows appearance of a yellowish-brown color in the reaction vessels which suggested the formation of colloidal Ag NPs. Excitation of surface plasmon vibration may be cause of this color change (Shahverdi *et al.,* 2007). Silver NPs synthesis within hours (*Fusarium oxysporum*) and minutes (*Aspergillus fumigatus*) were reported (Bhainsa and D'Souza, 2006). Intensity of color increased after 24h of incubation that resulted from high nanoparticles formation.

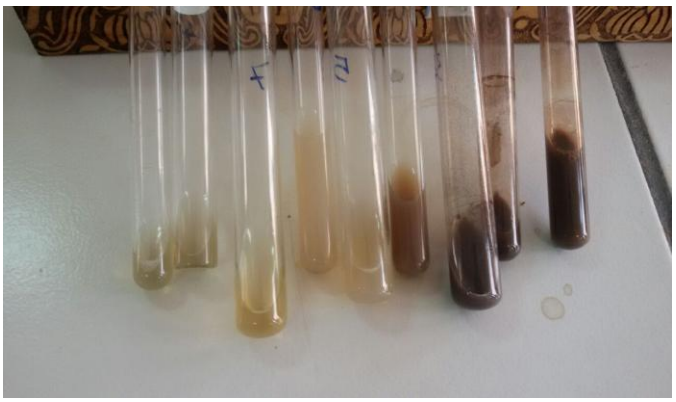


Fig. 1: Solutions of silver nitrate after exposure to the culture supernatant of *Bacillus* sp. HAI4 (from left to right 0.001, 0.01 and 0.1M).

The UV-Vis absorption spectra of the supernatant evaluated in the range of 300-800 nm by a double beam UV-Vis spectroscopy. Figures 2 show a strong broad absorption band located between 340 and 350 nm for silver NPs prepared by this bacterium. The absorbance in the range of 340-350 nm increases by increasing of $AgNO_3$ concentration.

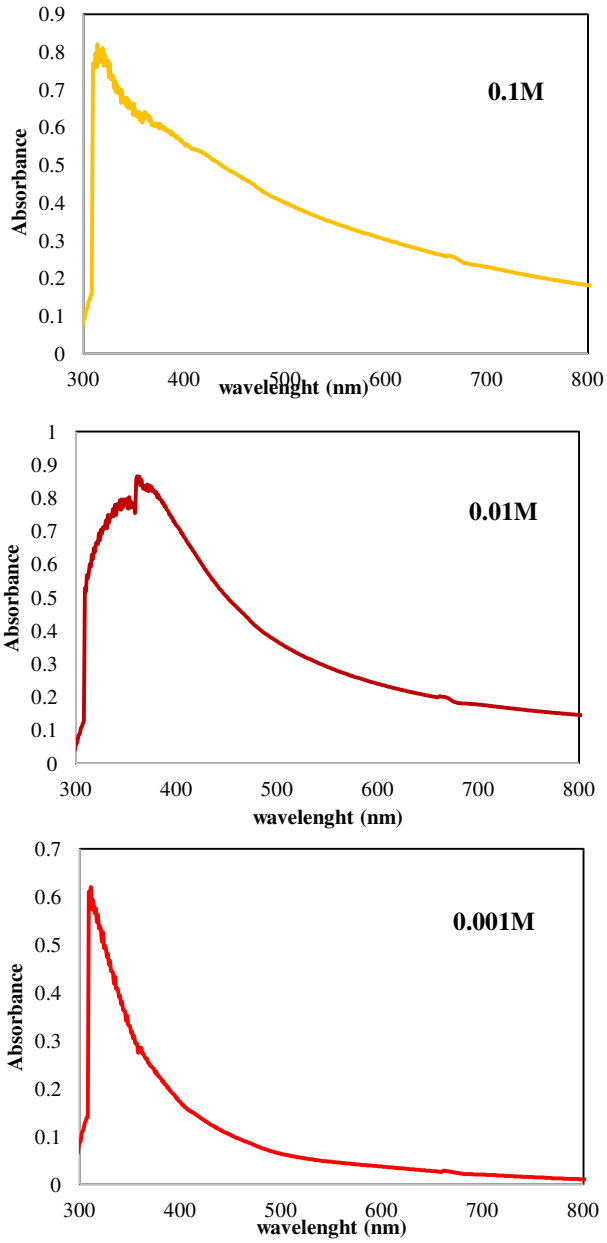


Fig. 2: UV-Vis spectrum of Ag NPs produced by *Bacillus* sp. HAI4 in three level of concentration 0.1, 0.01 and 0.001M.

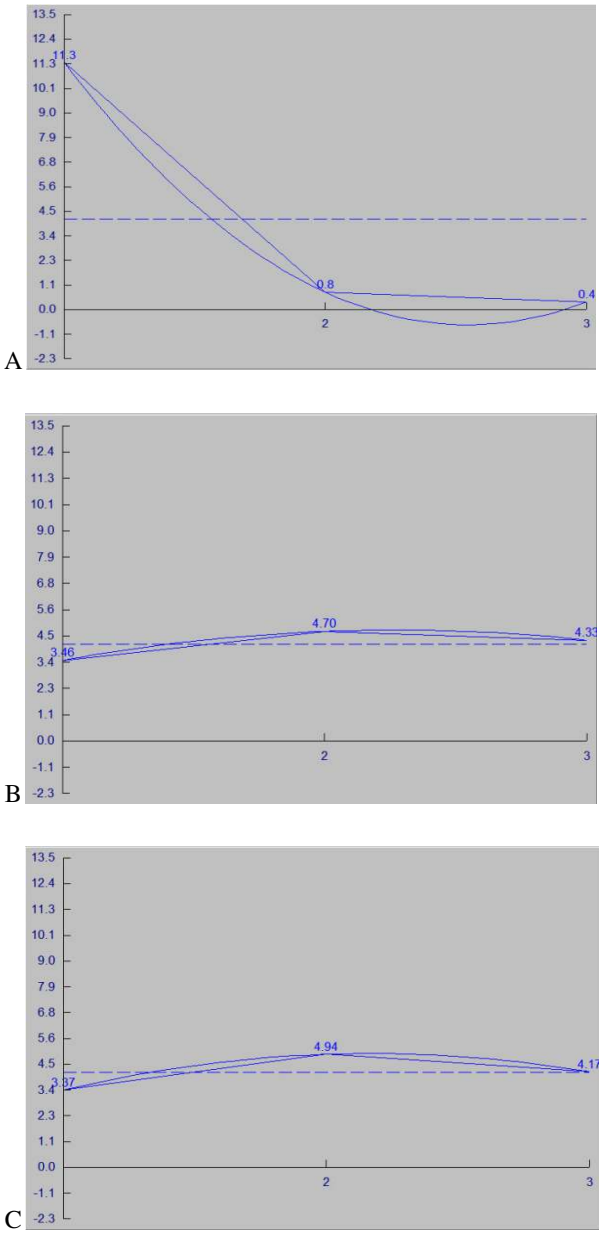


Fig. 3: Taguchi results of average effect of $AgNO_3$ concentration (a), incubation time (b) and culture time (c).

Table 1 illustrates effects of three different factors ($AgNO_3$ concentration, incubation time and culture time) on the silver NPs biosynthesis by *Bacillus* sp. HAI4. $AgNO_3$ concentration in level 1(11.326), incubation time in level 2 (4.695), culturing time in level 2 (4.172) had higher effect on the Ag NPs biosynthesis (Fig. 3).

Effective factors in silver nanoparticles synthesis by *Bacillus* sp. HAI4 are demonstrated by variance analysis. Final column determines each factors which major factor is $AgNO_3$ concentration.

Table 1: Effects of three different factors on the AgNPs biosynthesis.

Factors	Level 1 (value)	Level 2 (value)	Level 3 (value)
$AgNO_3$ concentration	11.326	0.793	0.361
Incubation time	3.457	4.695	4.327
Culturing time	3.37	4.94	4.172

Table 2: Analysis of variance for Ag NPs biosynthesis.

Factors	DOF(f)	Sum of Sqrs.	Variance	F-Ratio (F)	Pure Sum (S')	Percent (%)
$AgNO_3$ concentration	2	231.354	115.677	123.672	229.483	95.875
Incubation time	2	2.433	1.216	1.3	0.562	0.235
Culturing time	2	3.697	1.848	1.976	1.827	0.763

Table 3 shows optimum conditions for biosynthesis of Ag NPs affected by three factors. Expected result at optimum condition was 12.641% which is good result of biosynthesis by this bacterium.

Table 3: Optimum conditions of Ag NPs biosynthesis by bacterium.

Factors	Level	Contribution
$AgNO_3$ concentration	2	7.165
Incubation time	2	0.537
Culturing time	3	0.779
Total contribution from all factors	-	8.48
Current grand average of performance	-	4.16
Expected result at optimum condition	-	12.641

X-ray diffraction analysis

To confirm the crystalline nature of Ag NPs, further studies were done by X-ray diffraction (Fig. 4). This figure illustrates peaks 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 Ag NPs. Also, a few unmarked peaks were noticed in the vicinity of the characteristic peaks. These peaks can be resulted from the existing of capping agent stabilizing the NPs. The sizes of gained nanoparticles were estimated to be in the range of 33/264 nm using Debye-Scherer eq. (1):

$$d=k\lambda/(\beta \cos\theta) \quad (1)$$

where K, known as Scherer's constant, ranges from 0.9 to 1.0, λ is 1.5418 Å, which is the wavelength of the X-Ray radiation source, β1/2 is the width of the XRD peak at half height and θ is the Bragg angle (Fig. 4). XRD analysis showed the crystalline and amorphous nature of synthesized silver NPs. Analysis of SEM demonstrated that Ag nanoparticles were formed as aggregates and had spherical shapes (Fig. 5).

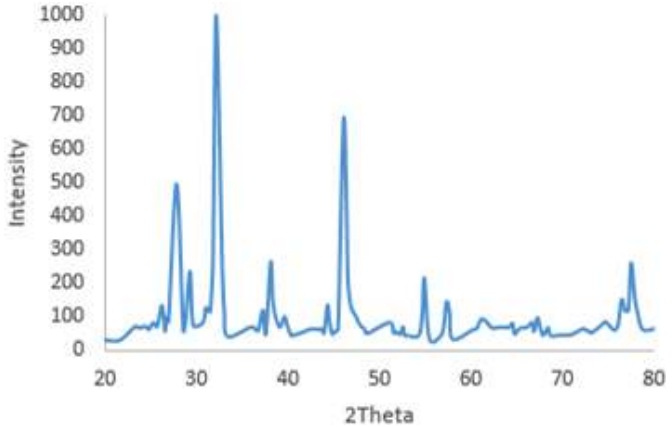
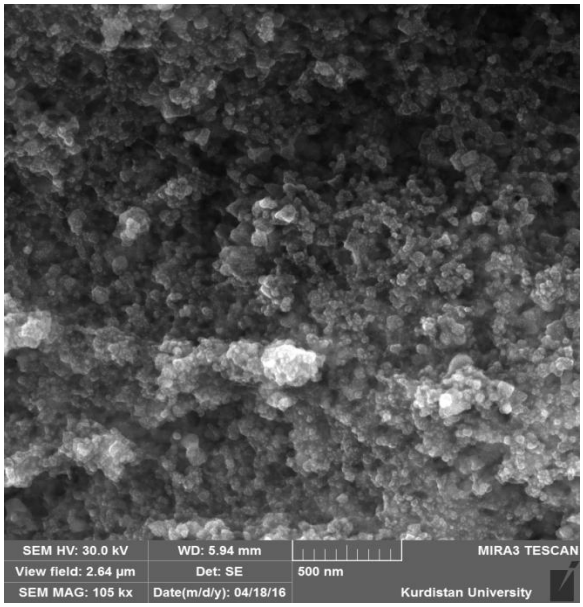


Fig. 4: X-ray diffraction spectrum of Ag NPs synthesized from 0.1 M $AgNO_3$ treated *Bacillus* sp. HAI4 cell free supernatant at 28˚C.



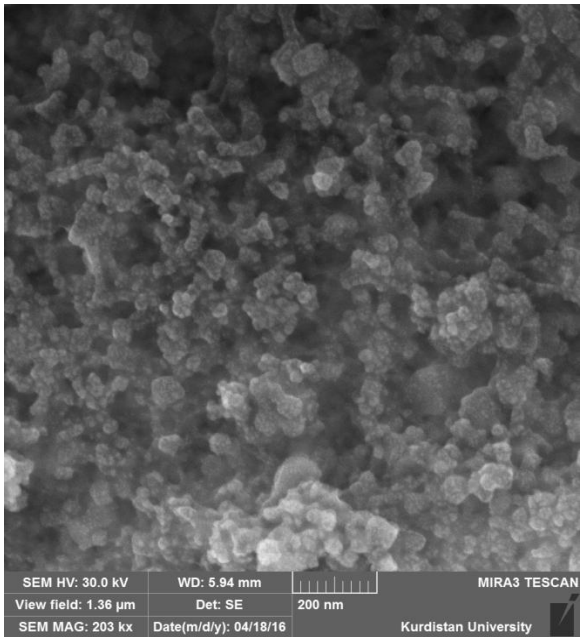


Fig. 5: Scanning electron microscopy (SEM) image of AgNPs produced by culture supernatant of *Bacillus* sp. HAI4.

FTIR analysis

This analysis demonstrates synthesized silver nanoparticles peaks at 834; 1,379; 1,634; 2,432; 2,972; 3,430 cm^{-1}. Three considerable bands could be observed from the figure 6. The intense peaks at 1379 and 1634 cm^{-1} corresponds to alkyl and C=C bonds respectively. The bond appearing at 3,430 cm^{-1} is assigned for O-H stretch of alcohol/phenol. FTIR spectroscopic analysis illustrated that culture supernatant of *Bacillus* sp. HAI4 has the ability to perform dual functions of reduction and stabilization of AgNPs. When the metal NPs produced in the solution, they must be stabilized against the Van der Waals forces of attraction which may otherwise lead to coagulation (Fig. 6).

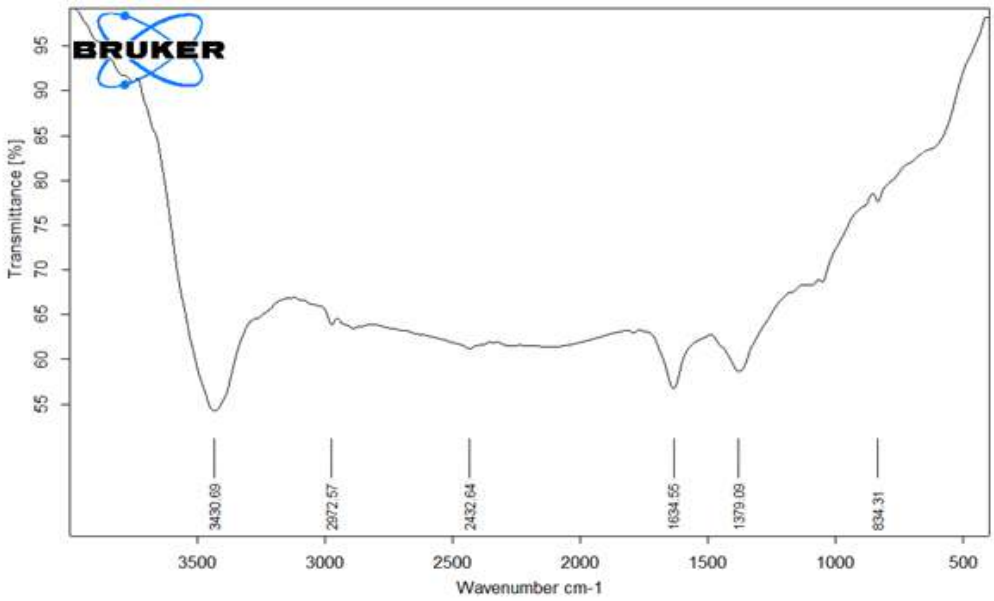


Fig. 6: FTIR spectra of AgNPs.

Antibacterial activity

Results of bactericidal effect of the nanoparticles on the pathogen bacteria are showed in table 4. In order to evaluating of antibacterial effects, maximum zone of inhibition, two important multidrug resistant pathogenesis bacteria, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 43300 were used (Feng *et al.,* 2000). Three levels of Ag NPs concentrations had antibacterial effect on these bacteria (1, 1 and 0.5 cm for *E.coli* and 1, 1.1 and 1.1 for *S.aureus*) but there was no significant difference between three levels (Fig 7).

Table 4: Antibacterial activity of AgNPs against *E.coli* ATCC 25922 and *S.aureus* ATCC 43300.

	Zone of inhibition (mm)		
Micro organisms	**0.1M**	**0.01M**	**0.001M**
Escherichia coli ATCC 25922	1	1	0.5
Staphylococcus aureus ATCC 43300	1	1.1	1.1

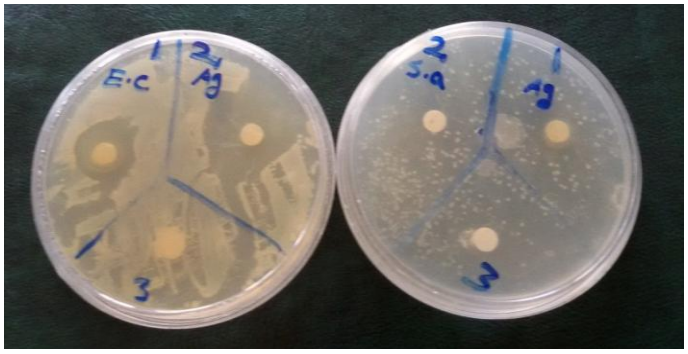


Fig. 7: Bactericidal activity of silver nanoparticles on *E.coli* ATCC 25922 (Left) and *S.aureus* ATCC 43300 (Right).

Antibacterial activity of Ag NPs is not merely resulted from their release of metal ions but also can be related to their morphology, specifically their small size and higher surface area to volume ratio. In this case, Guzman et al reported antibacterial activity of Ag NPs has been reported on the same bacterial strains (Guzman *et al.,* 2012).

CONCLUSION

There are many studies about biosynthesis of NPs by plant, fungi, and bacteria. In this work, silver nanoparticles synthesized by *Bacillus* sp. HAI4 extracellulary. Stabilizing of Ag NPs was possible without using any capping agents which are toxic.

This investigation shows major effect of $AgNO_3$ concentration on productivity of cell-free supernatant (12.641% as Expected result at optimum condition). This performance is very important in scale-up production of NPs synthesis. Also, these NPs have antibacterial effect that can be usable in medicinal aspect for fighting against prominent pathogen bacteria *E.coli* ATCC 25922 and *S.aureus* ATCC 43300. Sondi and Salopek-Sondi (2004) reported antimicrobial effect of silver nanoparticles on *E. coli* as a model of gram-negative bacteria. In total, this study presents simple, low expensive, eco-friendly and high productivity in fabrication of Ag NPs.

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