Chapter 3

Antibacterial Activity of Silver Species against *Escherichia coli* K12

3.1 Introduction

It is a well-known fact that silver ions and silver-based formulations (bulk and nano form) can be used as an antibacterial agents (Lemire et al., 2013). The nano form of silver possesses extensively high surface area to volume ratio as compare to its bulk form, which makes them more toxic for various microbial species. Ag NPs are known to be the most extensively used NPs in various consumer products because of their vast application as an antibacterial agent (Panáček et al., 2006; Durán et al., 2016). The physico-chemical properties of NPs are reported to be unique and tunable as compared to their bulk materials and majorly depends on their size, shape, crystallinity, and surface properties. (Agnihotri et al., 2014; Niska et al., 2016; Pareek et al., 2018).

Antibacterial property of Ag NPs can be significantly manipulated by variation in their physico-chemical properties. In addition, the antibacterial activity of Ag NPs also depends on the components of aqueous biological media in which their antibacterial activity is being tested. In antibacterial experiments, due to the presence of O_2 in aqueous biological media, the oxidative dissolution of Ag^+ ions from Ag NPs cannot be ignored. Ag NPs have been reported to show much higher bactericidal activity in oxygenic condition as compared to the anaerobic conditions (Lok et al., 2007; Xiu et al., 2012). This suggests that antibacterial action is due to the Ag^+ ions, and Ag NPs only act as reservoir and release Ag^+ ions due to their oxidative dissolution (McQuillan et al., 2012; Le Ouay and Stellacci, 2015). The rate of dissolution from nanoparticles can be regulated by various parameters like surrounding medium, physico-chemical properties of NPs, physical state of immediate environment (pH and ionic strength), etc., which ultimately makes a remarkable impact on their antibacterial activity of Ag NPs (Li et al., 2010; Kittler et al., 2010; Stebounova et al., 2011; Levard et al., 2013a). In addition, these parameters also decide the state of NPs, i.e., whether NPs will remain in mono-dispersed, aggregated or dissolved form in the aqueous biological media. Multiple reports are available on the effect of biological media components on Ag NPs and their dissolution rate (Table 3.1). However, the effect of various physico-chemical properties of Ag NPs on their interactions with biological media, bacterial cells, and precise cytotoxic action mechanism have not been explored in-depth. Hence, understanding the effect of physico-chemical modifications of Ag NPs towards variation in their antibacterial activity would be instrumental to elucidate the mechanism behind antibacterial action.

Table 3.1: Interaction of various components of biological media with Ag NPs and their effects on dissolution kinetics.

S. No.	Biological media component	Effect on Ag NPs	Rate of dissolution	Reference
1.	Ca^{+2} and Mg^{+2}	It allows the aggregation of NPs	Decrease	(Jin et al., 2010)
2.	Free thiol-containing groups (cysteine and glutathione)	These groups can bind on to the surface of Ag NPs and prevent the oxidation by inhibiting oxygen exclusion	Decrease	(Liu et al., 2010)
3.	Ascorbic acid	It behaves in a surprising way that at low concentration it increases the dissolution rate but at a concentration above 1 mM, completely inhibits the dissolution. This kind of activity is not understood completely but can be correlated to the generation of Reactive Oxygen Species (ROS)	Increase/ Decrease	(Liu et al., 2010)
4.	Chloride and phosphate ions	Precipitation of silver in the form of least soluble forms such as silver chloride and silver phosphate	Decrease	(Li et al., 2010; Levard et al.; 2013b; Loza et al., 2014)
5.	Sodium chloride	It allows the aggregation of NPs	Decrease	(Li et al., 2010; Levard et al., 2013b)

6.	Sulfide ion	Formation of an insoluble layer of Ag_2S occur on Ag NPs, thus rendering it to release Ag^+ and decrease the activity	Decrease	(Reinsch et al., 2012; Levard et al., 2013a)
7.	Glucose	Decelerate the rate of ion release from NPs because it is acting as a reducing agent	Decrease	(Loza et al., 2014)
8.	Hydrogen peroxide	Accelerate the rate of dissolution by forming peroxide intermediates	Increase	(Loza et al., 2014)
9.	Chlorine	Increases the release of ions in comparison to peroxide because it would be needed in very low concentration (2 mole of Ag NPs can be oxidized by only one mole of chlorine)	Increase	(Garg et al., 2016)

Considering the above-mentioned facts, the chemically and biologically synthesized Ag NPs with varied physico-chemical properties (discussed in Chapter 2) were evaluated for their antibacterial efficacy in comparison to silver ions. As Gram-negative bacteria are comparatively much resistant to various antibiotics due to the presence of their unique outer membrane (discussed in Chapter 1), in the present study gram-negative bacterium *Escherichia coli* K12 was used as a model organism.

3.2 Material and methods

3.2.1 Materials

All chemicals used for antibacterial studies were of analytical grade and purchased from Sigma Aldrich (USA) unless otherwise stated. Milli-Q water was acquired from a Milli-Q Biocel water purification system manufactured by Merck Millipore (Merck KGaA, Darmstadt, Germany).

3.2.2 Bacterial growth curve

Escherichia coli K12 (MTCC 1302) was purchased from Institute of Microbial Technology (IMTech), Chandigarh, India and revived from glycerol stock by culturing in Luria Bertani (LB) broth medium (casein enzyme hydrolysate 10 g L^{-1}, Sodium chloride 5 g L^{-1}, yeast extract 5 g L^{-1}, pH 7.2 ± 0.2) for 12 h at 37°C under shaking conditions (150 rpm). Considering the previous reports on precipitation of Ag^{+} ions in LB media by the presence of Cl^{-} ions of sodium chloride salt (Chambers et al., 2013; Bhargava et al., 2018), we compared the growth of bacteria in LB media and modified LB (MLB) media (without sodium chloride). Overnight grown bacterial culture (~3×10^{7} cfu mL^{-1}) was inoculated separately in freshly prepared LB and MLB broth medium (casein enzyme hydrolysate 10 g L^{-1}, yeast extract 5 g L^{-1}, pH 7.2 ± 0.2). The bacterial growth rate was determined by measuring the OD at 600 nm against blank (no bacterial cell) at 2 h time intervals (0.1 absorbance corresponds to a concentration of 10^{7} cfu mL^{-1}). The experiments were done in both technical & biological duplicates and the results are represented as mean ± standard deviation (SD). The growth curve of bacteria in LB and MLB broth medium were compared.

3.2.3 Dissolution kinetics of Ag NPs

In order to check the dissolution kinetics of Ag^{+} ions from NPs, equal concentration [10 µg (Ag) mL^{-1}] of differently sized (5, 10, 20, and 50 nm) and differently capped Ag NPs (C-Ag NPs, F-Ag NPs, and L-Ag NPs) were separately added in 10 mL of MLB broth medium in 50 mL Erlenmyer flasks. The flasks were incubated at 37°C under dark conditions at 150 rpm. The dissolution kinetics of Ag^{+} ions was quantified at 2, 4, 6, and 8 h by inductively coupled plasma optical emission spectrometry (ICP-OES) (Avio 200, PerkinElmer, USA) and the results are represented as mean ± SD of biological duplicates.

3.2.4 Antibacterial tests

The minimum inhibitory concentration (MIC) assay was performed to determine the lowest concentration of silver species (Ag^+ ions and Ag NPs of different size and surface capping) required to inhibit the growth of *E. coli* K12 using broth microdilution method as per the standard guidelines (CLSI, 2015). For MIC assay, the bacterial cells of mid-log phase were diluted in MLB broth medium to get a final cell density of ~3×10^7 CFU mL^{-1}. Initially, the bacterial cells were exposed to the broad range (two-fold dilutions) of all silver species (0.5, 1, 2, 4, 8, 16, 32, 64, 128, and 256 µg (Ag) mL^{-1} concentrations) in a final volume of 2 mL in 12-well untreated clear polystyrene flat bottom plates. The concentrations of all the NPs were calculated based on the concentration of silver present in the precursor salt (mentioned in Chapter 2) used during the NPs synthesis (Bhargava et al., 2018). The plates containing bacterial cells with NPs were incubated at 37°C under dark conditions at 150 rpm till the completion of log phase (8 h). The bacterial growth was determined by measuring OD at 600 nm. Based on the results obtained in broad range experiment, further experiments were performed using narrow range concentrations of silver species to find out the exact MIC value. Media without silver species and bacterial cells were used as positive and negative controls, respectively. In addition, the free form of surface capping agent (at similar concentrations and state used in the synthesis of Ag NPs) was also tested for their antibacterial activity. The experiments were done in both technical & biological duplicates and the results are represented as mean ± SD.

Based on the MIC assay results, Ag NPs which showed best antibacterial activity was further analysed for the determination of minimum bactericidal concentration (MBC) in comparison to Ag^+ ions. For MBC assay, the bacterial cells of mid-log phase were diluted in MLB broth medium to get a final cell density of ~3×10^7 CFU mL^{-1}. The assay was performed in the 12-well untreated clear polystyrene flat bottom plates with a final volume of 2 mL. Initially, the bacterial cells were exposed to the broad range (two-fold dilutions) of L-Ag NPs [0.5, 1, 2, 4, 8, 16, 32, and 64 µg (Ag) mL^{-1} concentrations] and incubated at 37°C under dark conditions for 24 h. Following exposure, 100 µL of bacterial sample was spread plated on the freshly prepared LB agar (1.5 %) plates. The plates were incubated at 37°C for 24 h to observe any growth. Based on the results obtained in broad range experiment, further experiments were performed using narrow concentration range of L-Ag NPs to find out the exact MBC value. Media without L-Ag NPs and bacterial cells were used as positive and negative controls, respectively (Bhargava et al., 2018).

3.3 Results and discussion

3.3.1 Bacterial growth kinetics

LB broth medium is a complex medium used for the cultivation of *E. coli* (Deininger, 1990; Sezonov et al., 2007). Biological medium components can make significant difference in the antibacterial activity of silver species (Table 3.1). The behaviour of Ag species (Ag^0 NPs/ Ag^+ ions) in the aqueous biological medium in which their antibacterial activity is tested, needs to be explored. In biological media and buffers with high salt content (chloride and phosphate), the particles may aggregate which leads to a decrease in the degree to which they can associate with bacterial cells (Lok et al., 2007). In LB media, presence of Cl^- ion of sodium chloride salt, which maintain tonicity of media, has been reported to cause precipitation of Ag^+ ions in the form of silver chloride (McQuillan et al., 2012; Chambers et al., 2013). This may result in the reduced availability of free Ag^+ ions and thus, reduction in the bactericidal activity. In order to overcome the precipitation issue, we have compared the growth of *E. coli* K12 in LB media and MLB media lacking sodium chloride, based on our previous report (Bhargava et al., 2018).

The bacterial growth kinetics was analysed by taking OD_{600} at 2 h time interval for 26 h. The bacterial growth curve represents the different phases (lag, log, stationary, and death) of bacterial population over a period of time in batch culture. Lag phase is the adaptation phase for bacteria to the media conditions; log phase is the period of rapid cell growth, stationary phase shows presence of growth limiting factors in media and thus no further acceleration in the bacterial population; and death phase is the period where bacteria starts dying due to lack of nutrients (Zwietering et al., 1990; Paulton, 1991). The obtained growth curve (Figure 3.1) showed the absence of lag phase in case of LB media as the young growing bacterial cells were used as inoculum and transferred to fresh LB media with same compositions. Interestingly, no lag phase was observed in case of MLB media (lacking NaCl) as well. In both media types, the duration of log phase was found to be 8 h. A non-significant difference was observed in the overall growth pattern of *E. coli* K12 in both media types. Based on these results, MLB media was chosen for the further antibacterial studies.

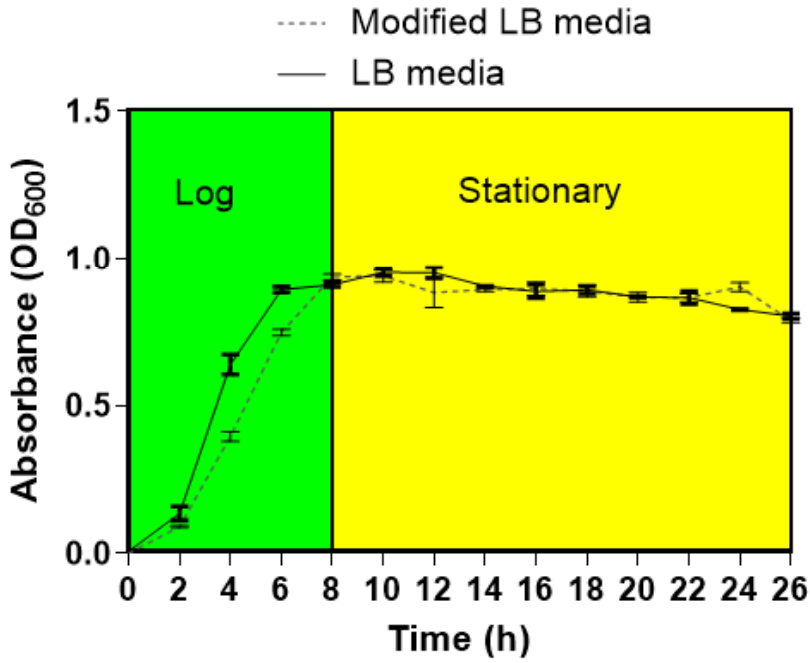

Figure 3.1: Growth curve analysis of *E. coli* K12 in LB and modified LB medium. Vertical bar represents SD.

3.3.2 Dissolution kinetics of Ag NPs

In aerobic conditions, Ag NPs undergo oxidative dissolution which tends to release silver ions in the surrounding medium, which regulates their antibacterial activity. The dissolution kinetics of silver ions get affected by the variations in the physico-chemical properties of Ag NPs (Pareek et al., 2018). We have checked the dissolution kinetics of silver ions from the synthesized Ag NPs of varied size and surface capping with respect to time up to 8 h.

A time dependent increase in the concentration of released silver ions was observed for all the sized Ag NPs (Figure 3.2 A). An increase in the size of NPs was found to be inversely proportional to the dissolution rate of silver ions. Ag NPs with an average particle size of 5 nm were found to have maximum dissolution rate of silver ions. After incubation of 8 h, 5 nm Ag NPs were found to have 66.78, 140.20, and 245.65 % higher dissolution of silver ions as compared to Ag NPs having average particle size of 10, 20, and 50 nm, respectively. Many previous reports have shown that size of NPs is inversely proportional to the dissolution of ions from NPs surface, which eventually affect their antibacterial potential (Ma et al., 2011). Moreover, surface capping also makes significant change in the dissolution kinetics of ions from the NPs (Niska et al., 2016). To find out the effect of citrate, fucose, lysozyme, and fungal proteins capping on the dissolution kinetics of synthesized Ag NPs, release of silver ions was

observed at regular intervals up to 8 h under experimental conditions. As expected, a time dependent increase in the concentration of silver ions was observed for all the tested Ag NPs during the incubation period of 8 h (Figure 3.2 B) (Lu et al., 2013; Agnihotri et al., 2014). Noteworthy, L-Ag NPs was found to have maximum dissolution of silver ions and showed 75.61, 150.41, 39.72, and 59.29 % higher dissolution of silver ions as compared to C-Ag NPs, F-Ag NPs, fungal protein-capped Ag NPs synthesized by *Penicillium shearii* AJP05, and *Penicillium janthinellum* DJP06, respectively.

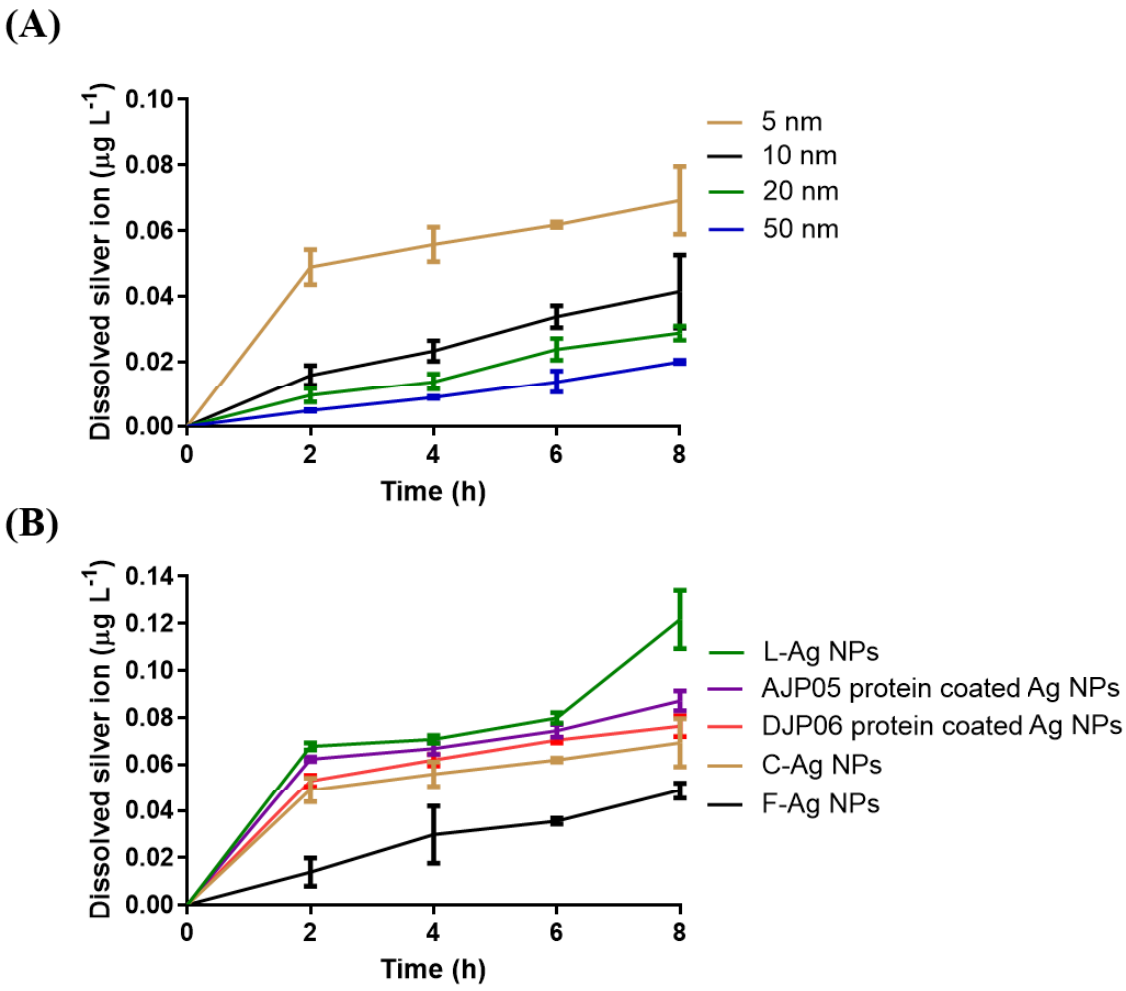

Figure 3.2: Dissolution kinetics of silver ions from the surface of Ag NPs with varied (A) size and (B) surface capping, with respect to time. Vertical bar represents SD.

3.3.3 Antibacterial assay

For the analysis of antibacterial activity, freshly grown *E. coli* K12 culture was exposed to different concentrations of silver species and observed for growth till the completion of log phase as per our previous report (Bhargava et al., 2018). Similar kind of work was reported by Pelletier et al. (2010), where antibacterial activity of cerium oxide NPs was determined against *E. coli* and *Bacillus subtilis* till the period of log phase (8 h). Likewise, Chatterjee et al. (2011) also determined the growth kinetics of *E. coli* till the completion of log phase, under the exposure of gold and Fe_3O_4 NPs.

3.3.3.1 Minimum inhibitory concentration (MIC) assay

Antibacterial activity of synthesized Ag NPs was analysed by MIC assay. The effect of size and surface capping of Ag NPs were evaluated in comparison to silver ions.

(A) Effect of variation in size of Ag NPs

To check the effect of Ag NPs size on their antibacterial potential, Ag NPs of various sizes (5, 10, 20 and 50 nm) synthesized by the chemical reduction method (discussed in Chapter 2) were assessed for their MIC against *E. coli* K12 in comparison to Ag^+ ions. The MIC of Ag NPs was found to be in the range of 33 to 214 µg (Ag) mL^{-1}. The antibacterial activity of NPs was found to be size dependent. The MIC value of Ag NPs with a size of 5, 10, 20, and 50 nm was found to be 33, 62.5, 124, and 214 µg (Ag) mL^{-1}, respectively (Figure 3.3). An increase in the size of Ag NPs leads to decreased antibacterial activity. This decrease in the antibacterial activity could be due to difference in the dissolution rate of silver ions from Ag NPs (Figure 3.2 A). Small sized NPs have been reported to dissolve more rapidly in comparison to large sized NPs and therefore possess higher antibacterial activity (Agnihotri et al., 2014; Pareek et al., 2018). With decrease in particle size, there will be simultaneous increase in the surface area to volume ratio of individual particle as well as in the relative particle concentration (Lok et al., 2007). Small sized NPs have excess energy at their surface due to the increase in their band gap energy. The properties are attributed to lattice contraction favour the confinement of surface electron which enhance their direct interactions with bacterial cells (Agnihotri et al., 2014). Ma et al. (2011) reported the size dependent dissolution of silver ions from Ag NPs. They used gum arabic (GA) stabilized Ag NPs of two different sizes (6 and 25 nm) as well as PVP coated Ag NPs with a size range of 5, 8, 25 and 38 nm. Based on the obtained results, they concluded that the increase in the size of NPs reduces the surface area to volume ratio that ultimately affect the dissolution rate of ions and antibacterial efficacy of the NPs. Size is one

of the most crucial parameter to decide the rate of dissolution because it gives the proper activity of NPs based on their accurate unit of mass or mole. Similar type of results were obtained by Lu et al., (2013), where, Ag NPs with an average size of 5, 15, and 55 nm were synthesized by the chemical reduction method and analysed for their antibacterial potential against both aerobic and anaerobic bacterial species. Agnihotri et al. (2014) also reported the size-specific antibacterial efficacy of Ag NPs synthesized using the co-reduction method.

The MIC of Ag^{+} ions was found to be 6 µg (Ag) mL^{-1}, which was much lesser in comparison to even the lowest sized Ag NPs (Figure 3.4). This suggest that in biological media, ionic form of silver become readily available to act on the bacterial cells. Ouay and Stellacci (2015) also reported that the antibacterial action is actually due to Ag^{+} ions, and Ag NPs only act as a reservoir and release Ag^{+} ions due to their oxidative dissolution.

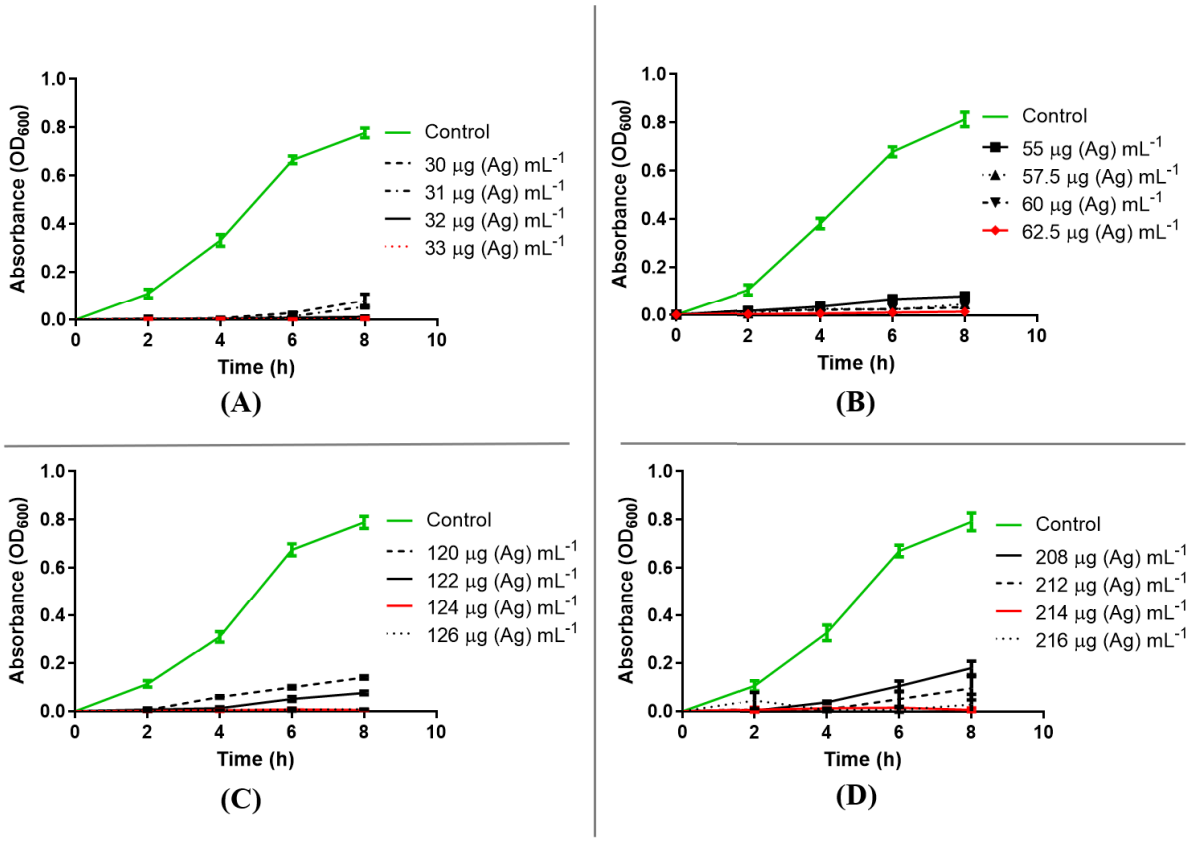

Figure 3.3: MIC analysis of chemically synthesized Ag NPs with an average size of (A) 5 nm, (B) 10 nm, (C) 20 nm, and (D) 50 nm. Vertical bar represents SD.

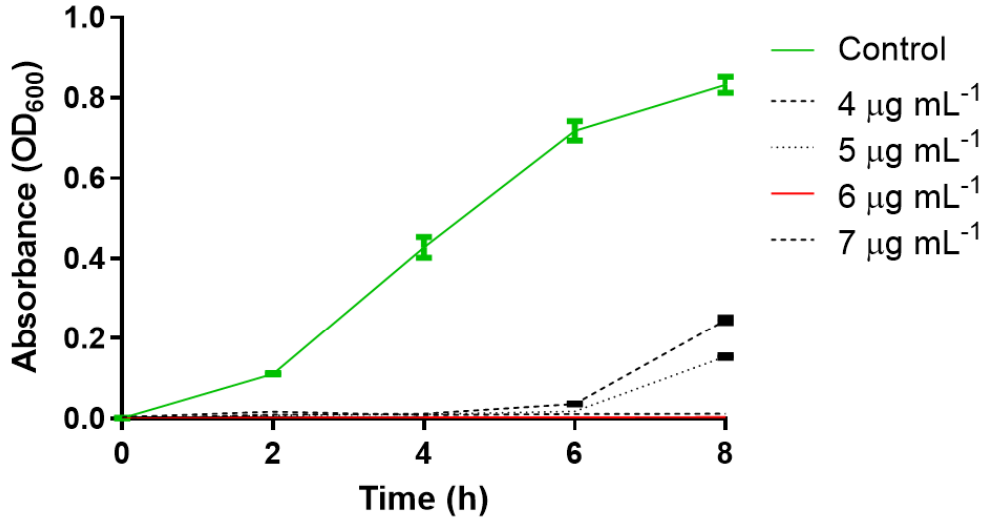

Figure 3.4: MIC analysis of silver ions. Vertical bar represents SD.

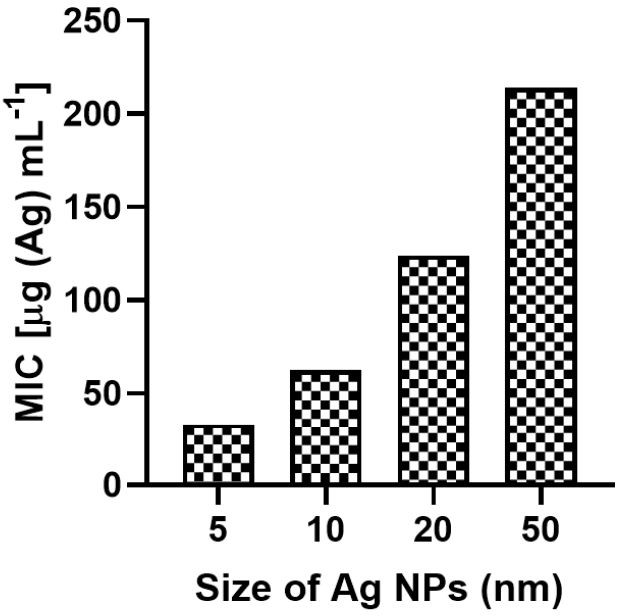

Figure 3.5: Comparative MIC of chemically synthesized Ag NPs with varied sizes.

Based on the results obtained from the MIC analysis of Ag NPs with varied size, small sized Ag NPs showed best antibacterial potential (Figure 3.5). Hence, Ag NPs of smaller size range (5-20 nm) were selected to study the effect of various surface coating agents towards their antibacterial potential.

(B) Effect of surface capping agents

Among various metals, silver has been known for its bactericidal activity since ancient time. However, the use of silver as a medicine has not yet been commercialized. Even Ag NPs, which have been reported as better antibacterial candidate due to their tuneable physico-chemical properties, failed to gain any commercial biomedical importance. The major reason for this is the unwanted toxicity of silver in the host cells witnessed during the antimicrobial applications (Bhargava et al., 2018). Use of suitable surface capping agent can modulate the release of Ag^{+} ions from the surface of Ag NPs as well as the interaction of Ag NPs with bacterial cells.

As discussed earlier, based on the best antibacterial activity, Ag NPs of smaller size range (5-20 nm) were selected to study the effect of various surface coating agents. Ag NPs with various surface coatings viz. citrate (C-Ag NPs), fucose (F-Ag NPs), lysozyme (L-Ag NPs) and fungal proteins were analysed for their antibacterial potential by the MIC assay. C-Ag NPs, F-Ag NPs and L-Ag NPs showed a MIC value of 33, 62.5 and 9 µg (Ag) mL^{-1} (Figure 3.6).

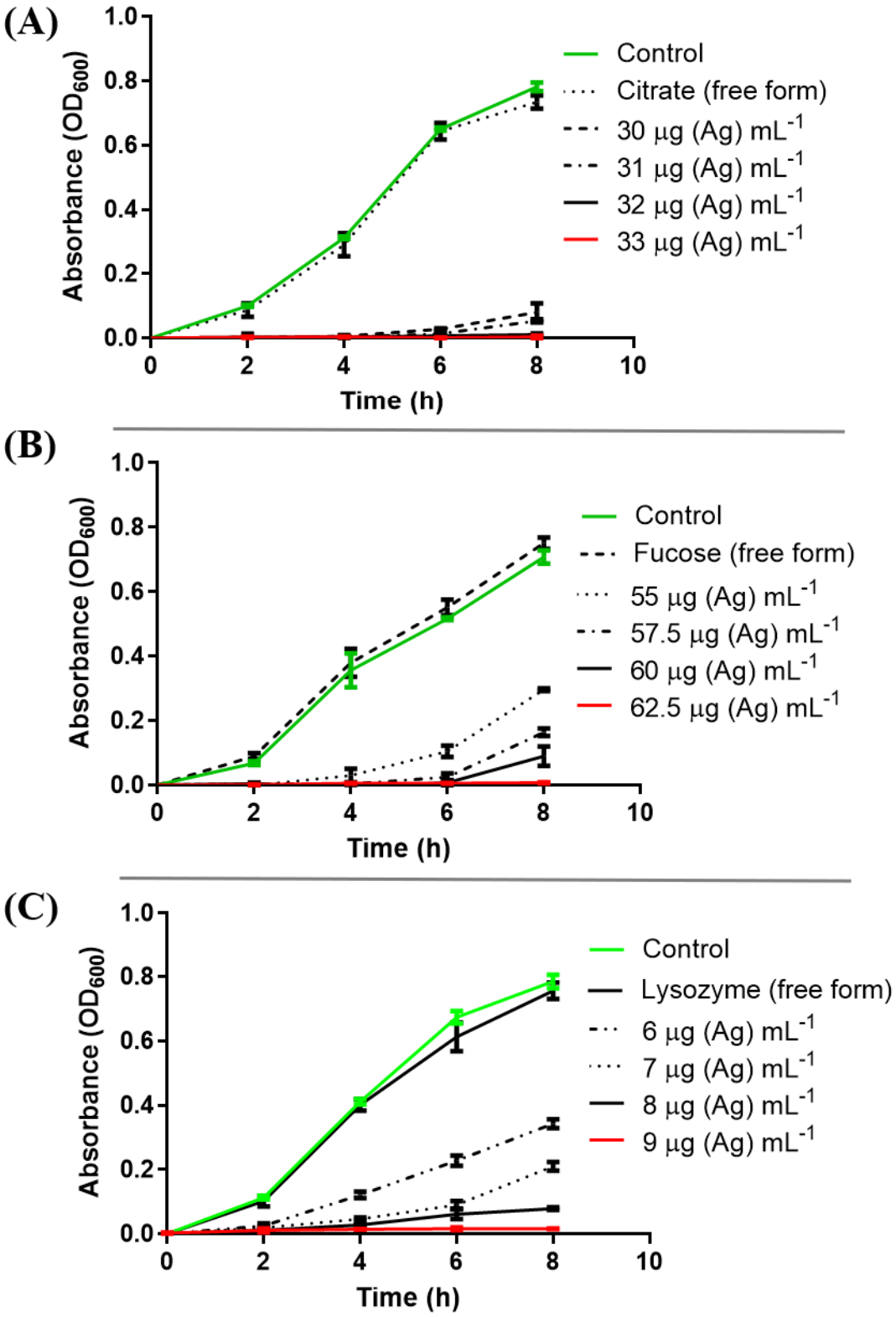

Figure 3.6: MIC analysis of chemically synthesized Ag NPs with variation in surface coating viz. (A) citrate, (B) fucose and (C) lysozyme. Vertical bar represents SD.

In order to rule out the possibility of bactericidal activity of citrate, fucose, and lysozyme, their free form (at similar concentration and state used in the synthesis reactions) were also tested. The obtained results suggested that, individually these capping agents did not show any antibacterial activity. Table 3.2 depicts the summarized MIC of chemically synthesized Ag NPs with varied size and surface capping agents.

Table 3.2: MIC of chemically synthesized Ag NPs with variation in their size and surface capping.

Physico-chemical variation	**Size of Ag NPs (nm)**	**Capping agent**	**MIC [µg (Ag) mL^{-1}]**
Size	5	Citrate	33
	10	Citrate	62.5
	20	Citrate	124
	50	Citrate	214
Surface capping	~5	Citrate	33
	~20	Fucose	62.5
	~5	Lysozyme	9

The MIC of biosynthesized Ag NPs was found to be 20 and 30 µg (Ag) mL^{-1} for the Ag NPs synthesized by *Penicillium shearii* AJP05 and *Penicillium janthinellum* DJP06, respectively (Figure 3.7). However, only fungal proteins did not show any antibacterial activity. The obtained results indicate that the antibacterial activity is solely due to the Ag NPs and the surface capping agents play an important role in facilitating the interaction of Ag NPs with the bacterial cells.

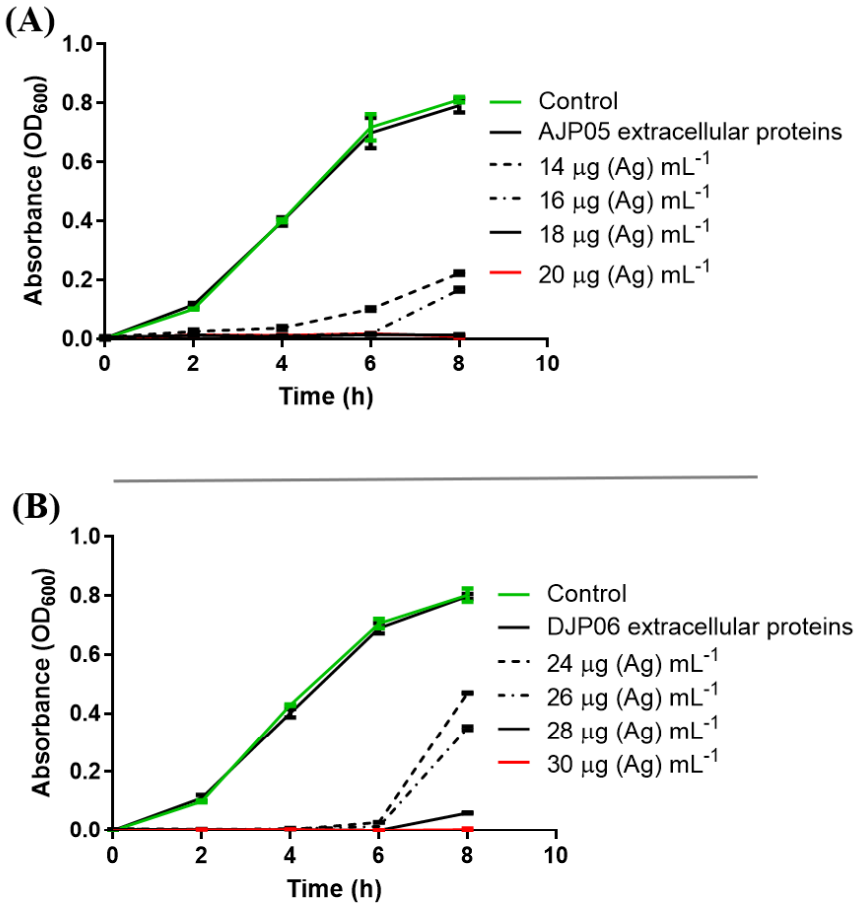

Figure 3.7: MIC analysis of Myco-synthesized Ag NPs by (A) *Penicillium shearii* AJP05 and (B) *Penicillium janthinellum* DJP06. Vertical bar represents SD.

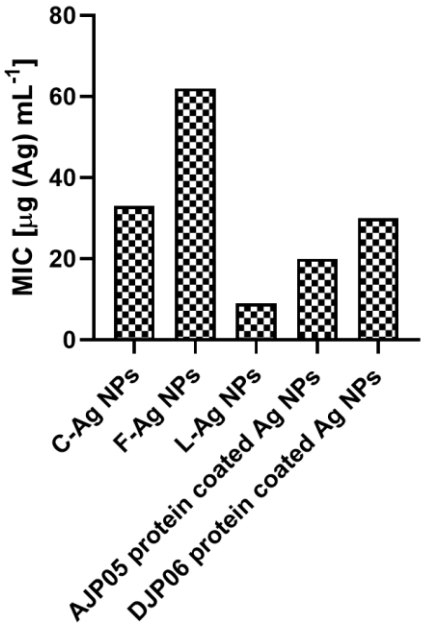

Figure 3.8: MIC comparison of Ag NPs with variation in their surface capping.

Figure 3.8 depicts the comparative MIC of chemically and biologically synthesized Ag NPs with varied surface capping agents. L-Ag NPs showed best antibacterial potential with lowest MIC value among all the tested Ag NPs. It is not feasible as well as not correct to compare the obtained MIC values with other studies due to large variation in various factors involved in the antibacterial studies such as varied initial bacterial concentration, bacterial strains, and media composition (Agnihotri et al., 2014).

Capping agent dependent antibacterial activity of Ag NPs has been well reported (Lu et al., 2013; Jain et al., 2015; Niska et al., 2016). Walczyk et al. (2010) advocated that instead of the shape and size of NPs, the cellular response to the NPs depends on the structure and composition of surface capping agents as this is what the cell sees first. The varied antibacterial potential observed during the present study can be due to the difference in the interaction of tested Ag NPs types with the *E. coli* K12 cells. C-Ag NPs can interact with bacterial cell by creating an electrostatic interaction between the negatively charged citrate moiety of C-Ag NPs with the positively charged residues of integral outer membrane proteins of bacteria (Eby et al., 2009; Agnihotri et al., 2014), thus releasing the silver ions in the proximity to the bacterial cells.

Earlier, we have demonstrated the superior antibacterial activity of F-Ag NPs against *Pseudomonas aeruginosa* PAO1 (gram-negative bacteria) in comparison to C-Ag NPs (Bhargava et al., 2018). The fucophillic "LecB" is one of the noteworthy lectin protein present in the outer membrane as well as cytoplasm of *P. aeruginosa*. This protein plays a prominent role in the attachment of the bacterial cell to the host tissues, promote bacterial cell self-aggregation, and biofilm formation. The specific association of LecB with the fucosyl residues was the main motivation for us to explore its applicability in increasing the antipseudomonal potential of the Ag NPs. The availability of lectins in the *E. coli* fimbriae, further convinced us to test these Ag NPs against *E. coli* K12. However, contrasting results were obtained with *E. coli* K12 used in the present study as F-Ag NPs showed MIC value of 62.5 in comparison to the MIC value of 33 obtained with C-Ag NPs. This could be due to the absence of LecB in the outer membrane of *E. coli*, which was necessary for the close interaction with F-Ag NPs as observed in case of *P. aeruginosa*. The absence of close electrostatic interaction between F-Ag NPs and *E. coli* cells could have resulted in lesser antibacterial efficacy of F-Ag NPs in comparison to the C-Ag NPs.

In general, lysozyme is not an active bactericidal compound against the gram-negative bacteria because of the presence of extra outer membrane layer. However, addition of hydrophobic peptide to the C-terminus of lysozyme makes it lethal for the gram-negative bacteria, which suggest that hydrophobic region of lysozyme facilitates its interaction with the bacterial cell (Ibrahim et al., 1992; Ibrahim et al., 1994; Ravindran et al., 2013; Ashraf et al., 2014). Increase in the hydrophobicity of lysozyme can also be achieved by its thermal denaturation at 80°C for 20 min. rather than addition of hydrophobic peptides to the C-terminus. At 80°C, lysozyme is reported to convert from monomeric to dimeric form having a hydrophobic structural motif which actually plays a pivotal role in its interaction with bacterial cells, thus, becomes lethal to gram-negative bacteria (Ibrahim et al., 1996). However, in the present study, during the synthesis of L-Ag NPs (as discussed in Chapter 2), lysozyme was treated at much higher temperature (120°C) for 1 h time duration, which leads to the complete denaturation of lysozyme. Hence, the free form of lysozyme could not show any antibacterial activity (Figure 3.6 C). During the synthesis of L-Ag NPs, the treatment at 120°C for 1 h duration could have exposed the active hydrophobic motifs of lysozyme which then allowed close interactions of L-Ag NPs with the bacterial cells, and thereby, enhanced their antibacterial potential.

Apart from the interactions with bacterial cell, surface coating also makes a terrific impact on the ion release kinetics from Ag NPs. Damm and Münstedt (2008), studied the effect of polymer on the dissolution rate of Ag NPs by forming polymer/ silver nanocomposites. They used different range of polyamides [polyamide (PA) 6 grade, PA 6.6 grade, PA 12 grade, PA 12 modified with poly-THF grade and cycloaliphatic PA 1] to form nanocomposites and noted the release of silver ions in following manner: PA12 < cycloaliphatic PA < PA12-poly-THF < <PA6. 6 < PA6. This pattern of dissolution was due to the increased water content of polymer, which made the difference in the coating of Ag NPs. In the present study, variations in the dissolution kinetics of Ag^{+} ions were observed from the surface of Ag NPs with varied surface coatings (Figure 3.2 B). The obtained results were found to be in accordance to the observed MIC values. Based on the MIC assay, a schematic presentation of possible mechanism for the antibacterial action of Ag NPs with varied surface capping is speculated (Figure 3.9).

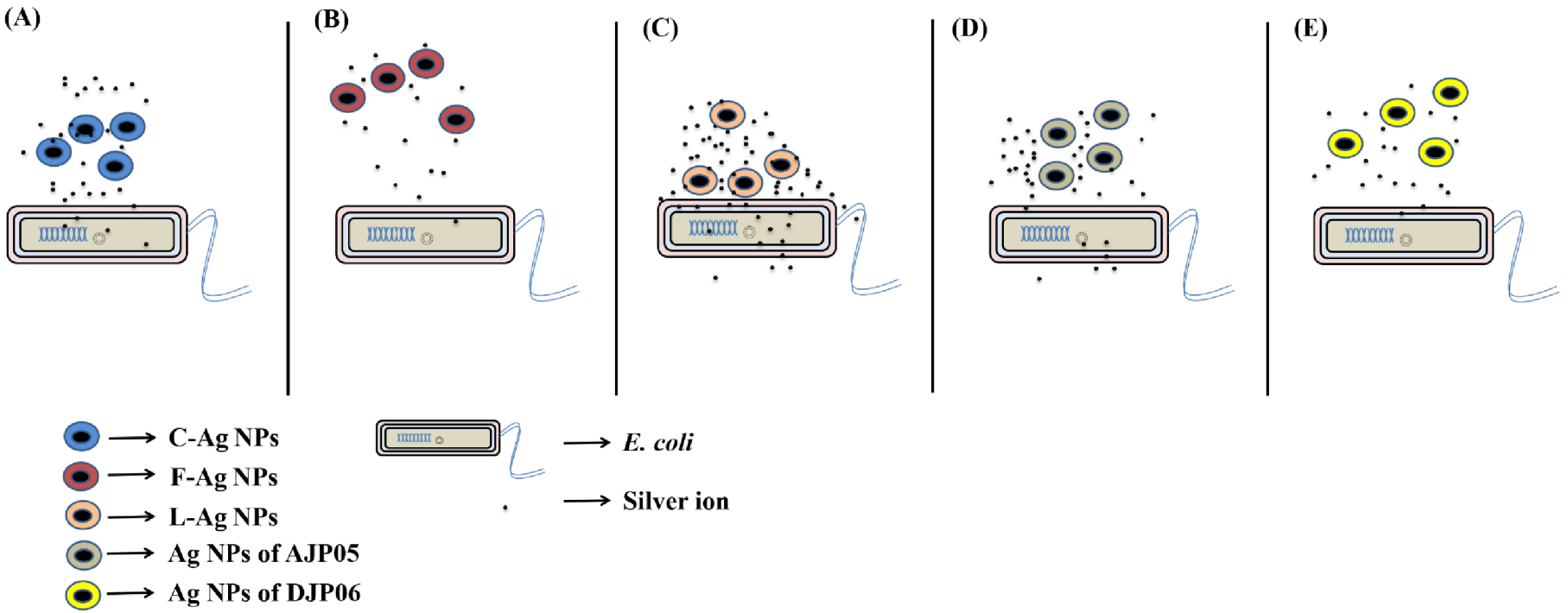

Figure 3.9: Schematic presentation of possible mechanism for the antibacterial action of Ag NPs with varied surface capping

3.3.3.2 Minimum bactericidal concentration (MBC) assay

MBC assay was performed to find out the lowest concentration of silver species (Ag^+ ions/ Ag NPs) needed to kill 99.9 % of the final inoculum after incubation for 24 h under standardized conditions (CLSI, 2015). As L-Ag NPs showed the best antibacterial potential with lowest MIC value among all the tested Ag NPs, they were analysed for determination of MBC in comparison to Ag^+ ions. The bacterial cells were treated with different concentrations of L-Ag NPs followed by spread plating 100 µl of exposed bacterial cells on LB agar plates. The lowest concentration of L-Ag NPs causing bactericidal effect was selected based on the absence of visual bacterial growth on agar plates and reported as MBC. Representative images of MBC analysis for verifying concentrations of L-Ag NPs have been shown in Figure 3.10, which confirmed the MBC of L-Ag NPs to be 30 µg (Ag) mL^{-1}, whereas MBC for silver ions was found to be 18 µg (Ag) mL^{-1} (Figure 3.11).

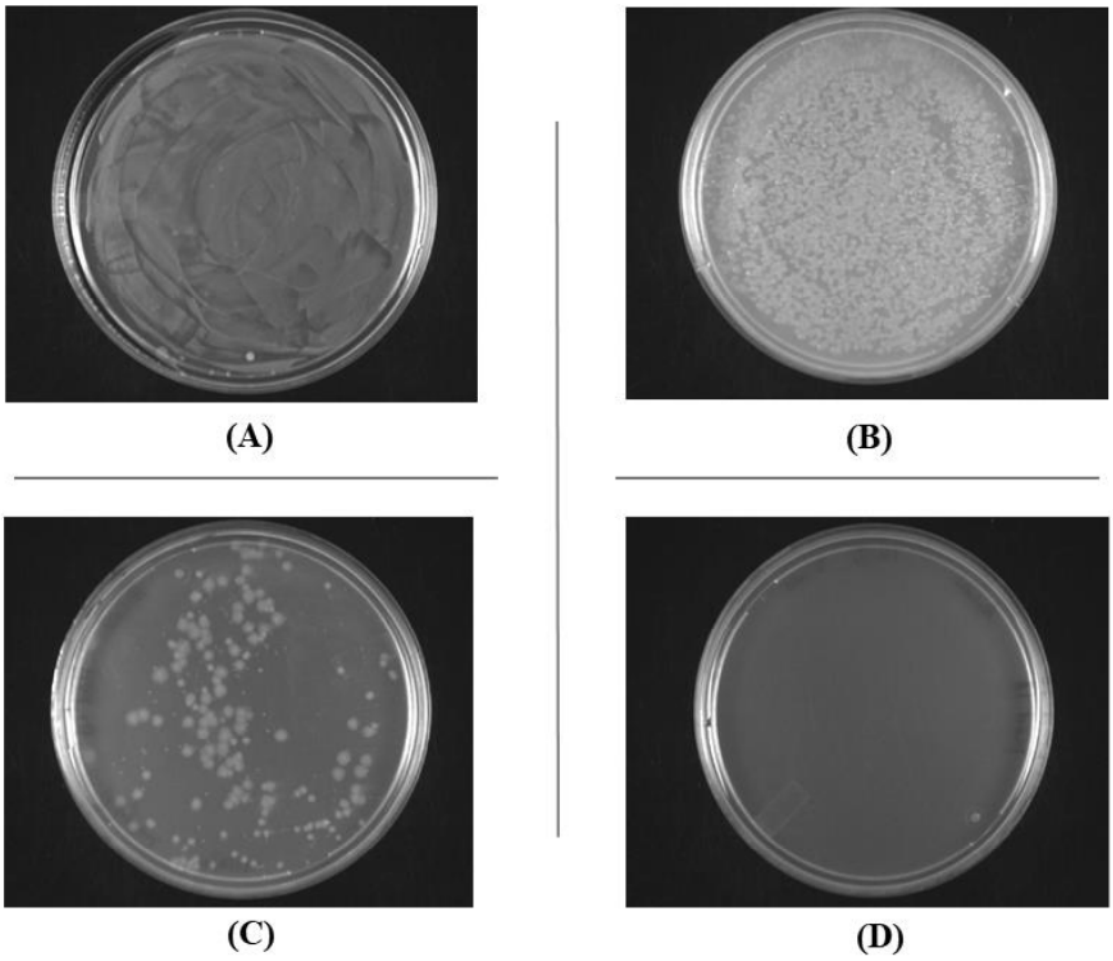

Figure 3.10: MBC analysis for verifying concentrations (A) 0 (Control), (B) 28, (C) 29, and (D) 30 µg (Ag) mL^{-1} of L-Ag NPs against *E. coli* K12.

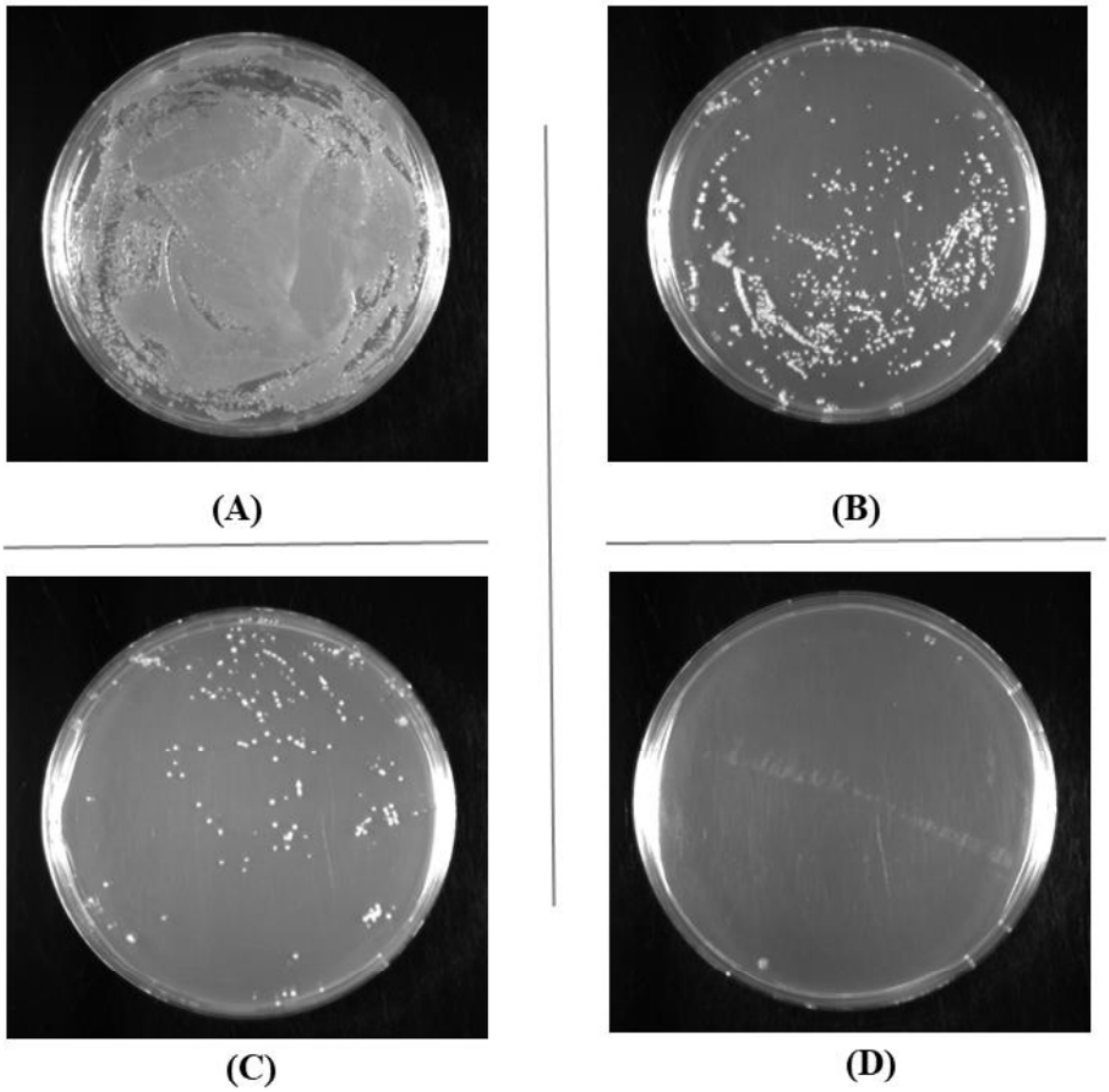

Figure 3.11: MBC analysis for verifying concentrations (A) 0 (Control), (B) 16, (C) 17, and (D) 18 µg (Ag) mL^{-1} of silver ions against *E. coli* K12.

As depicted in Figure 3.10 and 3.11, exposure of both the silver species (Ag^{+} and L-Ag NPs) adversely affected the bacterial viability. The MBC/MIC ratios were calculated to determine the presence or absence of tolerance to silver species. A bacterium is known to be tolerant to any antibacterial agent, when the MBC/MIC ratio value is found to be ≥ 32 (Sader et al., 2006; Traczewski et al., 2009; Gonzalez et al., 2013). The MBC/MIC ratio for L-Ag NPs and silver ions was found to be 3.33 and 3, respectively. Since no tolerance was exhibited, it suggested that L-Ag NPs and silver ions can be used as an alternative to standard therapy for bacterial infections.

3.4 Conclusion

The present chapter deals with the evaluation of antibacterial potential (MIC) of chemically and biologically synthesized Ag NPs with varied size and surface capping agents in comparison to silver ions ($AgNO_3$) against gram-negative model organism *E. coli* K12. Ag NPs with an average size of ~ 5 nm showed best antibacterial activity with a MIC of ~ 33 µg (Ag) mL^{-1}. An increase in the size of Ag NPs leads to decreased antibacterial activity. A negative correlation was observed with the increase in size of NPs and the dissolution kinetics of Ag^+ ions from them. L-Ag NPs showed the best antibacterial activity with a MIC of 9 µg (Ag) mL^{-1}. Higher dissolution of silver ions from L-Ag NPs suggested the controlled and sustainable release of silver ions from their surface which subsequently participate in antibacterial action. Based on the best antibacterial activity of all the tested Ag NPs types, L-Ag NPs were selected for further studies on the mechanistic insights of antibacterial activity in comparison to silver ions.