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Bacterial Inactivation Kinetic Studies Using Biosynthesized Silver Nanoparticle

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ABSTRACT

Nanotechnology is a growing field in the biomedical sciences for its large surface volume ratio and effective applications. The silver nanoparticle is emphasized for its antibacterial efficiency. The study focuses on the inactivation of microorganisms using silver nanoparticle. The silver nanoparticle was synthesized in a time-saving manner the from silver-resistant bacterial strain Pseudomonas putida. For isolate silver-resistant strain, Pseudomonas putida was screened using an optimized concentration of 1mM silver nitrate-supplemented medium. After the incubation of 24 h, the resultant Pseudomonas putida was observed with the accumulation of silver ions in their cytoplasmic region. The amount of silver ion accumulated in Pseudomonas putida was calculated by Atomic Absorption Spectroscopy as 0.048 (±0.002) mg/L. The reduction of accumulated silver ions into silver nanoparticle was monitored using UV spectroscopy at 420 nm. The synthesized silver nanoparticle was effectively extracted by a modified cloud point extraction method. The optimized concentration of 1% Triton X-100 was used for the maximum extraction of silver nanoparticle. The synthesized silver nanoparticle was quantified by Inductively coupled plasma-optical emission spectrometry and found to be 0.350 (±0.005) mg/L. The SEM morphological analysis confirmed the presence of spherical silver nanoparticle and their average particle size was measured using particle size analyzer as 57 nm. The antibacterial efficiency of silver nanoparticle in different concentrations was studied using the shake flask method and the direct plate count method. The results were observed with the complete growth inhibition of E.coli at 0.5 ppm. The experimental studies on E.coli by silver nanoparticle show prominent log reduction and morphological denaturation. The inactivation potential of silver nanoparticle against bacteria was described by the inactivation kinetic models using Chick, Chick-Watson, and Homs.

Key words: Antibacterial, Cloud Point Extraction, Direct plate count, Growth curve, Lagging, Log reduction, Pseudomonas putida, Silver Resistant

INTRODUCTION

Nanoparticle are nanoscale molecules with intriguing physicochemical properties and are utilized for a variety of applications in a different fields. The nanoparticle possesses a large surface ratio than the bulk material improves the modalities and is more reactive (Krishnakumar et al. 2015). Silver nanoparticle pave more attention due to their Plasmon resonance activity (Anthony et al. 2014). The silver nanoparticle is considered a potential antimicrobial agent and minimizes the risk of using physical and chemical disinfectants (Mao et al. 2013). Silver nanoparticle are also used in medical devices, diagnosis ships, wound dressing bandages, drug delivery systems, optical sensors, semiconductors, etc., (Thamilselvi and Radha 2017).

Increasing demand for silver nanoparticle insisted on large-scale synthesis by an eco-friendly, stable method using biological systems. The physical reduction method for the synthesis of silver nanoparticle including evaporation, condensation, and laser ablation, have the disadvantage of discharging enormous heat and occupying more area. Whereas the chemical reduction method of silver nanoparticles emits carcinogenic gases to the environment and toxic sludge. Moreover, both physical and chemical methods of preparing silver nanoparticle highly depend on their physical environmental conditions, higher energy consumption, high maintenance, and expensive (Kulkarni et al. 2015; Suchomel et al. 2015).

The alternative synthesis of silver nanoparticle using biological systems from plants, microbes, etc., are considered an eco-friendly method and has evidence in different industrial application (Muthukrishnan et al. 2015; Baker et al. 2015; Gupta et al. 2015; Otari et al. 2015). Bacterial synthesis of silver nanoparticle was reported as an advantageous method due to its ease of handling (Vaidyanthan et al. 2010). The silver nanoparticle synthesis was reported after 3 days from algal culture, the cell-free microalgal extract (Sharma et al. 2015). The fungal synthesis of silver nanoparticle after 72 h

and the bacterial synthesis of silver nanoparticle from Pseudomonas deceptonensis was reported after 72 h (Vigneshwaran et al. 2007; Jo et al. 2016). The reported studies concluded that the biological synthesis of silver nanoparticle is a time consuming process.

Various extraction process of silver nanoparticle from biological sources was reported by sedimentation field flow fractionation, centrifugation, and separation using magnetic particle (Mwilu et al. 2014). Cloud Point Extraction is one of the rapid, easy, and environmentally friendly methods of extracting silver nanoparticle from their aqueous medium. Cloud Point Extraction is the liquid-liquid extraction process and the separation processes depend on the clouding temperature of the surfactant and the alteration of the experimental parameters such as pH, temperature, time, and concentration (Cacho et al. 2016).

The successful separation of silver nanoparticle using Triton X-114 from municipal wastewater was reported (Roy et al. 2016). Triton X-114, is the non-ionic and hydrophobic surfactant reported for the separation process (Everberg et al. 2006).

Therein we reported the rapid synthesis of silver nanoparticle from silver resistant bacterial strain Pseudomonas putida and the recovery of synthesized silver nanoparticle using cloud point extraction using Triton X-100. The modified extraction using Triton X-100 is an attempt to get a stable silver nanoparticle, because the bioactive silver is stabilized using the protein particles are not denatured by Triton X-100. The antibacterial efficiency of the silver nanoparticle is experimented against E. coli.

MATERIALS AND METHODS

Chemical reagents of an analytical grade were purchased from Sigma Aldrich and used. The standard cultures of Pseudomonas putida NCIM 2650 and Escherichia coli 2685 were procured from the National Collection of Industrial Microorganisms (NCIM), Pune, India. The cultures were maintained at 4˚C on a Luria Bertani slant for the purpose of subculture and the glycerol stock culture of bacterial strains were maintained at -80˚C. The experimental inoculum preparation a loopful of bacterial colonies were inoculated in a LB broth and kept in a shaker flash (150 rpm) at 37 ºC. The cell suspensions were diluted with a sterile saline solution to obtain a final experimental concentration of ~10^5 cfu/ mL, by comparing with a 0.25 McFarland turbidity standard, by taking $O.D_{600}$.

Screening of Silver Resistant Pseudomonas Putida

The silver resistant strain of Pseudomonas Putida was screened for rapid synthesis of silver nanoparticle. The standard subculture of Pseudomonas Putida NCIM 2560 was inoculated into a LB agar plate supplemented with silver nitrate of varying concentrations of 0.5 mM, 1 mM, and 1.5 mM. The culture plates were incubated at 37ºC for 24 h and observed for the silver resistance colonies surviving on the plates. The maximum silver nitrate concentration tolerable by the silver resistant Pseudomonas Putida was taken as the maximum threshold concentration and used for further experimental studies.

Synthesis of Silver Nanoparticle

For the rapid synthesis of silver nanoparticle, a loopful silver resistant Pseudomonas Putida was inoculated into 250 mL of LB broth supplemented with silver nitrate. The maximum threshold concentration of silver nitrate was supplemented for the experimental flask and incubated at 37ºC. The control experiment was done by inoculating the silver resistant Pseudomonas Putida without silver nitrate supplementation and maintained in the same experimental condition. The experiments were performed in triplicates for reliability. The synthesis of silver nanoparticle was inferred by the colour change that occurred in the experimental flash with respect to time.

Extraction of Silver Nanoparticle

The synthesized silver nanoparticle in the aqueous phase was separated using cloud point extraction and the modified procedure of Nazar et al was as follows; To elucidate the optimum Triton X-100 for maximum extraction of silver nanoparticle, the sample was extracted with different concentrations of Triton X-100 (Nazar et al. 2011). The 10 mL of silver nanoparticle containing supernatant was taken into a centrifuge tube and added with 5 mL of triton X-100 in different concentrations of 0.5%, 1%, and 2%, and 0.1 mL of 3.5 mM $NaNO_3$. The sample solution pH was kept at 3, adjusted with 1 M HCl. The sample was kept in a water bath for 30 min at 65˚C, above the cloud point temperature of triton X-100. The inference of cloudy appearance in the sample centrifuge tube indicated the formation silver nanoparticle and triton X-100 aggregates. The samples were centrifuged (10000 rpm) for 10 min followed by the cloud point appearance. The upper aqueous phase was drained and the lower phase was washed several times with distilled water and 0.1 M HNO_3 in methanol. After washing, the silver nanoparticle was dried in an oven at 100˚C for overnight and stored for further study.

Characterization studies

Determination of Metal Accumulation

The silver accumulated on the silver resistant P. putida NCIM grown in different concentrations of 0.5 mM, 1 mM and 1.5 mM silver nitrate medium was quantified by Atomic Absorption Spectroscopy (AAS). The biomass from an overnight culture of silver resistant P. putida was obtained by centrifugation (10000 rpm) for 15 min. The supernatant was drained and the biomass was washed with distilled water. The biomass were kept for overnight drying at 65ºC. The dried cell mass was subjected to overnight acid digestion with 70% nitric acid. The digested samples were filtered using Whatman No.1 filter paper and the total volume of the sample was adjusted to 10 mL. The sample was measured in a 5% HNO_3 matrix using a Perkin-Elmer Analyst 300 AAS mounted with a silver lumina cathode lamp of Perkin-Elmer SK.

Quantification of Synthesized Silver Nanoparticle

The silver nanoparticle extracted using different concentrations of Triton X-100 was separately taken and quantified by ICP-OES using Perkin Elmer Optima 5300 DV. The obtained silver nanoparticle after washing with distilled water and nitric acid in methanol to remove the triton and impurities was kept for overnight digestion with 70% nitric acid. The digested samples were filtered and quantified.

UV-Spectroscopy Analysis

The sample from the experimental flask was periodically withdrawn and the synthesis of silver nanoparticle was confirmed by the UV-Spectroscopic analysis (T 90+ UV/Vis SPEC PG Instruments Ltd.). The sample was observed spectroscopically in different wavelength ranges of 360-440 nm.

Scanning Electron Microscopy.

The morphology of the synthesized silver nanoparticle was analyzed by Scanning electron microscopy (Carl Zeiss MA15 / EVO 18). The powdered silver nanoparticle was dissolved in water and sonicated for 1 h for uniform dissolution of the sample. The dried film of the sample was prepared by coating a small amount of the sample by dropping it on a carbon coated copper grid. The sample film was subjected to coating and analyzed using SEM.

Particle Size Analysis.

The silver nanoparticle suspension was prepared by adding 0.1 mg of silver nanoparticles dispersed in deionized water. The sample was sonicated for 20 min and the particle size was analyzed using a particle size analyzer (Malvern zetasizer Nano-ZS, Malvern instruments, U.K). The sample was analyzed in triplicates at 25ºC at a scattering angle of 173º in a disposable sizing cuvette under laser light beams. The double distilled water was taken as a reference dispersing medium.

Interaction of Silver Naoparticle Against E. Coli

Membrane Destabilization Assay

To detect the membrane damage of E. coli cells by the interaction of silver nanoparticle was analyzed by membrane destabilization assay. The 24 h culture of E. coli grown in LB broth at 37˚C was withdrawn and ~ 10^5 CFU/mL of E. coli was incubated with 0.5 ppm of silver nanoparticle for 6 h in a shaker incubator. The control experiment without silver nanoparticle was done in the same environmental condition. The biomass from both treated and control samples was obtained by centrifugation (2000 rpm) for 30 min. The biomass samples were added with equal volumes of PBS (Phosphate Buffer Saline) and 0.15% of SDS (Sodium dodecyl sulfate). The samples were analyzed by taking $O.D_{600}$ for observing the viability of E. coli through turbidity.

Protein Denaturation Study Using Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The silver nanoparticles treated and untreated bacterial cells were sonicated in a Heicher (German) sonicator, and centrifuged at 10000 rpm for 15 min to remove the intact bacteria. The resulting supernatant was added with 85% of ammonium sulphate, and kept overnight, at 4°C. The ammonium sulphate added supernatant was centrifuged at 10000 rpm for 15 minutes at 4°C and the pellet was re-suspended in 10 mM phosphate buffer (pH 7.4) and dialyzed against distilled water. The samples were then subjected to protein analysis by SDS-PAGE (Liu et al. 2012) using the final acrylamide concentrations of 10% and 5% (w/v) for separating and stacking gels, respectively. After electrophoresis, the gel was fixed with a solution containing 15% ethanol and 1% acetic acid, and washed with distilled water for 4 h; then the gel was subjected to Coomassie brilliant blue (CBB).

DNA Denaturation Study by Agarose Gel Electrophoresis

The damage of E. coli DNA by the interaction of silver nanoparticle was analyzed by Agarose gel electrophoresis. The DNA from the treated and untreated bacterial culture was obtained by centrifuging at 5000 rpm for 5 min. The cell pellet was resuspended in 200 μL of TE buffer at the pH 8.0, and 50 ng of RNase was added to digest the contaminating RNA. Then, 400 μL of Solution containing 1%, w/v of Sarkosyl, 0.5 M NaCl, and 1%, w/v of SDS was

added to the sample. The sample was mixed well and incubated for 10 min at 37 ºC with intermittent shaking at every 5 min. Immediately, an equal volume of phenol: chloroform: isoamyl alcohol in the ratio of 25:24:1 was added and mixed by inversion. The suspension was centrifuged at 10,000 rpm for 5 min and 0.1 volume of sodium acetate (3 M, pH 5.2) and 0.6 volume of isopropanol were added. The suspension was centrifuged for 5 min at 10,000 rpm at 37 ºC, and the supernatant was discarded. The pellet was washed with 1 ml of 70% ethanol and centrifuged at 10,000 rpm for 3 min at 37 ºC. The supernatant was discarded and the pellet was air-dried and re-suspended in 100 µL of sterile TE buffer, and analyzed by Agarose gel Electrophoresis (Sobhy et al. 2016). After electrophoresis, the gel was visualized under a UV transilluminator and photographed.

Structural morphology of E. coli by Optical electron microscopic analysis

The denaturation of E. coli by silver nanoparticle was confirmed by treating the E. coli of mid-exponential growth phase with 0.5 ppm of silver nanoparticle and incubating for 6 h. Then the silver nanoparticle treated samples were withdrawn and centrifuged (4000 rpm) for 30 min. The pellets were fixed on the glass slide and stained with methylene blue. The specimen slide was observed in an Optical electron microscope (Nikon Eclipse 100) under a magnification of 100XA/0.90A.

Influence of silver nanoparticles on E. coli growth by shake flask method

The growth profile of E. coli (~10^5 cfu/mL) with the influence of different concentrations of silver nanoparticle from 0.1 to 0.5 ppm was analyzed by the shake flask method. The E. coli was allowed to grow in a culture flash containing LB broth supplemented with different concentrations of silver nanoparticle and the culture flasks were incubated in an orbital shaker at 37°C for 24 h. The control experiments without silver nanoparticle under the same environmental conditions were monitored. The samples were withdrawn at regular intervals of time (1 h), and their growth profile was monitored spectroscopically by taking $O.D_{600}$.

Log reduction of E. coli by Plate count method

The bactericidal activities of silver nanoparticle against E. coli was experimented by the direct plate-counting procedure. The E. coli concentration of ~10^5 cfu/mL was incubated in LB broth supplemented with different concentrations of silver nanoparticles (0.1 -0.5 ppm) at 37°C in a shaker incubator. The control was prepared under ideal conditions in the absence of silver nanoparticle. The culture sample was periodically withdrawn after 1h. The samples were diluted with LB broth 10^4 fold and were poured (2µL) into a nutrient agar plate and incubated at 37°C for 24 h. The direct plate count of viable colonies was used to analyze the bactericidal activity of silver nanoparticle.

where N_0 is the initial concentration of E. coli (m^{-3}), N is the concentration of E. coli at time t (m^{-3}), and k' is the inactivation rate constant (min^{-1}), n is the coefficient of dilution and m is the order reaction rate constant.

RESULTS AND DISCUSSION

Isolation of silver-resistant P. putida

Figure 1 (a), (b), and (c) showed the Psuedomonas putida NCIM 2560 inoculated in an agar plates supplemented with different concentrations of 0.5 mM, 1 mM, and 1.5 mM silver nitrate. After the incubation of 24 h, the agar plates supplemented with 0.5 mM and 1 mM concentration of silver nitrate were observed with the viable colonies of Pseudomonas putida resistant strain.

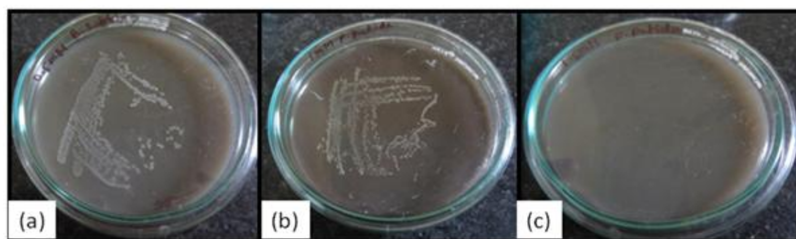

Figure 1 ***P. Putida*** **colonies on silver nitrate supplemented agar plates (a) 0.5 mM (b) 1 mM and (c) 1.5 mM**

At the concentration of 0.5 mM and 1 mM silver nitrate the Pseudomonas putida was able to accumulate the silver ions and replicate the resistant strains. The Pseudomonas putida inoculated in the agar plates supplemented with 1.5 mM concentration of silver nitrate was observed with the absence of noticeable colonies after 24 h of incubation, indicated that the bacterial strain was not able to tolerate and inhibits the bacterial growth. These results inferred that the

maximum concentration of silver nitrate tolerable for P. putida was 1 mM, taken as a maximum threshold concentration (Otari et al. 2015).

Synthesis of silver nanoparticles from silver resistant P. putida

Figure 2 shows the UV spectrum of silver nanoparticles synthesized from silver resistant P. putida NCIM 2650 at 48 h. Among the wavelength ranges from 360 to 440 nm, the broad and sharp peak was observed at 420 nm for the synthesis of silver nanoparticle. The absorbance at 420 nm was attributed to the surface plasmon region of silver nanoparticles (Kalimuthu et al. 2008).

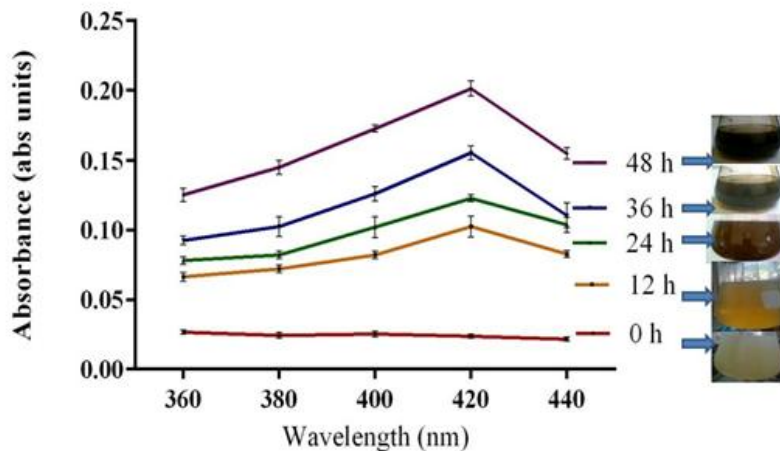

Figure 2 Silver Nanoparticles Synthesis from Silver Resistant P. Putida NCIM 2560

The absorption peak was observed to be increased in the range from 0.065 to 0.201 with the increasing incubation time of 12 h to 48 h respectively. This increasing absorbance peak with the increasing incubation time confirms the synthesized silver nanoparticles. However, after 48 h of incubation time, there was no change in the UV absorbance indicated the completion of the reduction process. Singh et al (2013) also reported about the increased synthesis of silver nanoparticles with the increase in incubation time (Singh et al 2013). The nitrate (silver nitrate) ions in the culture medium that enhances the synthesis of nitrate reductase, an enzyme that reduces the silver ion into silver nanoparticle (Gurunathan et al. 2009). After saturation of nitrate ions in the culture medium further reduction was not proceeded.

Quantification of the Accumulation of Silver on Pseudomonas Putida

The silver ions accumulated in the P. putida was observed to be increased as 0.032(±0.002) mg/L to 0.048(±0.002) mg/L with the increasing concentration of silver nitrate from 0.5 to 1 mM as shown in the Table. 1.

Table 1. Accumulated Silver on Silver Resistant P. Putida

Sl.No.	Concentration of silver nitrate (mM)	AAS quantification of silver on silver resistant P. putida (mg/L)
1.	0.5	0.032 (±0.002)
2.	1	0.048 (±0.002)
3.	1.5	ND

(ND- non detectable)

P. putida exposed to 1.5 mM concentration of silver nitrate was observed with the absence of noticeable silver accumulation, may be due to the intolerable bacterial cells not able to reproduce. The bacterial strains are able to accumulate metal ions at their tolerable concentration (Sedlak et al. 2012). The exopolysaccharides produced in the bacterial cell induces the metal uptake and accumulation of bacteria at a lower metal ion concentration (Deschatre et al. 2013). Increasing concentration of silver decreases the growth of an organism and leads to leathality (Deepak et al. 2011). Hence the optimum concentration of silver ions in the surrounding environment, may lead to the acclamitization

of E. coli to that and induce the accumulation of silver ions in the intracellular and the periplasm regions (Slawson et al. 1992; Klaus et al. 1999).

Extraction, Quantification, Morphological Studies of Silver Nanoparticle

The crude silver nanoparticle sample was taken in a tapered glass centrifuge and added with 0.5 mL of 1% Triton X-100 and 3.5 mM Sodium nitrate (Figure 3 a). Triton X100 is a non ionic surfactant uniformly dissolved in distilled water and 3.5 mM Sodium nitrate solution was used to maintain the ionic strength and the pH 3 was maintained by adding HNO3 to avoid metal ions hydrolysis (Kim et al. 2007). The sample was incubated in a water bath at 70ºC for 30 min, inferred with the cloudy appearance indicating cloud point formation (Figure 3 b).

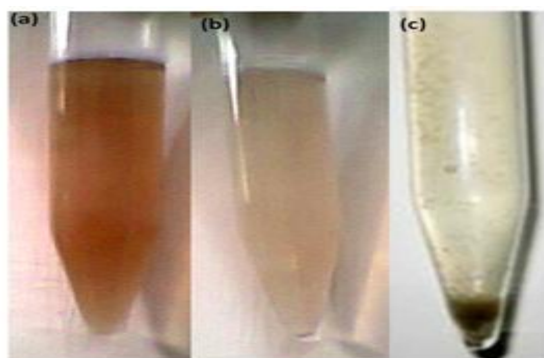

Figure 3 Extraction of Silver Nanoparticles (A) Sample Added with 1% Triton X-100 (B)Cloud Point Appeared (C) Separated Silver Nanoparticle Pellet

The sample was centrifuged (10000 rpm) and the upper aqueous phase was decanted and the lower surfactant rich phase with silver nanoparticle pellet was taken (Figure 3 C). The sample was washed with nitric acid in methanol solution followed by water washing. The resultant silver nanoparticle was dried in an oven and quantified. The silver nanoparticle was measured after extraction with different concentration of Triton X-100 as shown in Table.2.

Table 2 ICP-OES Quantification of Extracted Silver Nanoparticle

Sl.No.	Concentration of Triton X-100 (%)	Silver nanoparticle (mg/L)
1.	0.5	0.290 (±0.002)
2.	1	0.350 (±0.005)
3.	2	0.340 (±0.002)

The maximum of silver nanoparticle was extracted at the concentration of 1% of Triton X-100, taken as an optimum surfactant concentration for the effective extraction of silver nanoparticle. The results showed the presence of maximum silver 0.350 (±0.005) mg/L measured at the wavelength of 328.068 nm extracted using 1% Triton X-100.

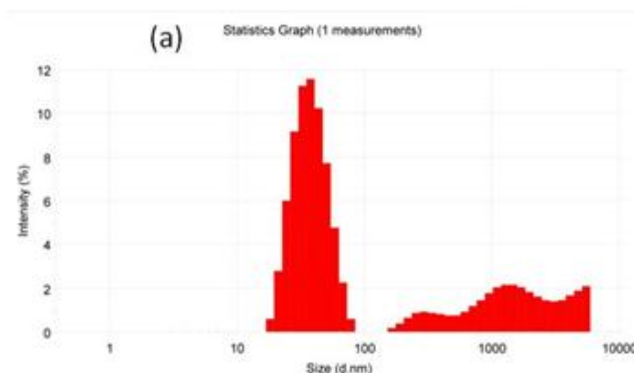

Figure 4 Paricle size distribution of silver nanoparticle

The SEM image shown in Figure 4, was observed with the cluster of spherical silver nanoparticle. The particle size analyzer results in the bar chart (Figure 5) confirmed the average size distribution of the silver nanoparticle as 57 nm.

Destabilization of E. Coli Membrane by the Interaction Of Silver Nanoparticle

The silver nanoparticle effectively interacted with the membrane surface active groups of E. coli and damaged the cell wall, were analyzed by their capacity to be lysed by the sodium dodecyl sulphate (SDS) treatment.s The silver nanoparticle treated E. coli cells and the control were observed for their viability by spectroscopically at $O.D_{600}$.

The silver nanoparticle treated E. coli cells were observed with a significant decrease in their turbidity than that of the control. The silver nanoparticle treated cells lost their permeability and became more vulnerable to SDS treatment and lysed. The control, treated with SDS was observed with no significant decrease in turbidity (Figure 6). SDS is a detergent which destroys the bacterial cells by disrupting their cell wall. SDS disrupts more rapidly the damaged bacterial cells that lose membrane stability due to any stress than the normal cells (Chamakura et al. 2011; Melo et al. 2013).

Silver Nanoparticle Damages E. Coli Protein

SDS- PAGE analysis of the protein profile for the control, E. coli without silver nanoparticle, and the silver nanoparticle treated E. coli were shown in Figure 7(1). The protein profile of the control is clear and broader. The cell growth in the control sample was higher and the protein yield was more, which was expressed by the broader clear band intensities. However, the silver nanoparticle treated E. coli protein profiles were the same as the control, but the band intensities were comparatively lesser than the control band, due to its growth inhibition. This indicates that the silver nanoparticle had a quantitative difference in protein content, but no qualitative effects on protein expression (Tiwari et al. 2008).

Silver Nanoparticle Damages E. Coli DNA

The silver nanoparticle interacted with the surface active groups of E. coli, that leads to cellular leakage and DNA Damage. The DNA damage of the silver nanoparticle treated E. coli was analyzed by agarose gel electrophoresis (Figure 7(2)). The DNA profile of the control, E. coli without silver nanoparticle was observed with dark and broader bands and the silver nanoparticle treated E. coli observed with the lighter band of the DNA. The lighter band was obtained due to the uncoiling of the DNA and broken DNA resultant activity of the silver nanoparticle (Meiwan et al. 2011).

Optical Electron Microscopy Of E. Coli

The Structural changes of E. coli cells exposed to silver nanoparticle were observed microscopically as shown in the

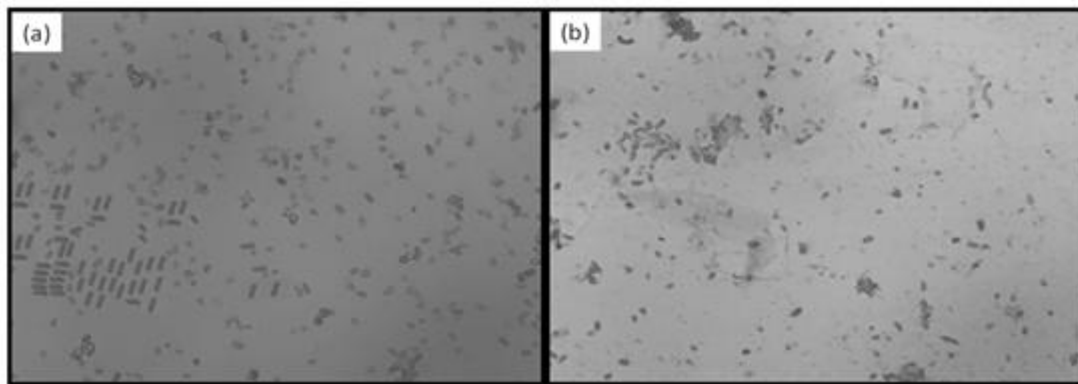

Figure 5 Microscopic images of E. coli (a) Before treatment; (b) After treatment

The damaged cells were observed as a cluster of cells with irregular shapes, and agglomerated with cellular extensions, whereas the control cells appeared normal rod shaped and intact. The silver nanoparticle treated E. coli cells were lost their membrane integrity due to the interaction of silver ions that leads to cellular contents leakage. This condition may lead to sublethal injury of E. coli, in which the cells fail to grow in a detectable range and in the nonculturable state than after sometimes the cells becomes lethal (Pal et al. 2007).

Effect of Silver Nanoparticles on E. Coli

The growth profile of E. coli in LB medium supplemented with different concentrations (0. 1 – 0.5 ppm) of silver nanoparticle was monitored by taking $O.D_{600}$ as shown in Fig. 9 (a). The growth profile of control experiment, E. coli

without silver nanoparticle was observed with distinct lag, exponential and stationary phases. The growth profile of silver nanoparticle treated E. coli showed the lagging curve, which was more prominent with the increasing concentration of silver nanoparticle. The growth profile of E. coli at a lower concentration of silver nanoparticle (0.1 ppm) was slightly lagged than that of the control (0 ppm); the increasing concentration of silver nanoparticle was observed with the prolonged log phase with growth delay. At the silver nanoparticle concentration of 0.5 ppm was observed with complete growth inhibition of E. coli. The results inferred that the silver nanoparticle possess the inhibitory effects on bacterial growth. The silver nanoparticle concentration and contact time are the influencing factor for the growth of E. coli, and the increased concentration of silver nanoparticle was observed with complete inactivation (Melo et al. 2013).

The E. coli inactivation by direct plate count assay for different concentration (0.1 - 0.5 ppm) of silver nanoparticle was analyzed by measuring their viable colonies and plotted against incubation time as shown in Figure 9 (b). From the results, it was concluded that the complete growth inhibition of E.coli was achieved at 0.5 ppm concentration of silver nanoparticle. The E. coli inactivation rate is higher with the increase in disinfectant concentration and increased incubation time (Al Hakami et al. 2013).

The percentage reduction of E. coli after treatment with different concentrations of silver nanoparticle (0.1-0.5 ppm) was determined by calculating viable cell count CFU/mL at periodic time intervals using the equation (1) (Jung et al. 2008):

$$Reduction\ \% = \frac{(N_0 - N_t)}{N_0} X\ 100 \qquad (1)$$

where N_0 is the initial number of CFU/mL and N_t is the number of CFU/mL at any defined time. The percentage reduction of E. coli against the incubation time was plotted as shown in Figure 9 (c). The results showed the percentage reduction was higher at the concentration of 0.5 ppm of silver nanoparticle and the inactivation time after 60 min was observed with 40 % of E. coli reduction. The complete inhibition of E. coli were achieved at 540 min. The increasing disinfectant concentration and increasing treatment time had positive control on bacterial inhibition (Li et al.2010; Xiong et al. 1999).

The Log reduction of E. coli after treatment with different concentrations of silver nanoparticle were shown in Figure 9 (d). The log reduction rate increases from 1.6 $\log_{10}$ CFU/mL (0.1 ppm) to 7.01 $\log_{10}$ CFU/mL (0.5 ppm) with the increased concentration of silver nanoparticle. The E. coli cells exposed to silver nanoparticle leads to survival stress. To counter this condition, the bacterial cells, lagging their growth and adapting themselves to that condition are physiologically active but metabolically inactive state. The metabolically inactive cells are failing to grow to a detectable level (Li et al. 2010). Further exposure of silver nanoparticle causes sublethal injury to the bacterial cells, and they are unable to produce viable colonies. The log reduction may be due to the lethality of E. coli (Xiong et al. 1999).

Bacterial Inactivation Kinetics

The kinetic models were used to understand the complex inactivation of E. coli by silver nanoparticles as shown in Figure 6 (Table 3). The Homs model was observed with significant R^2 values (>0.9) in all concentrations.

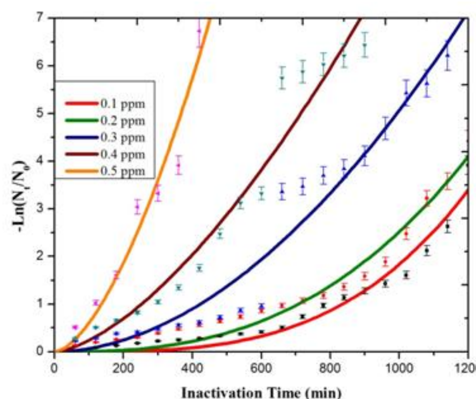

Figure 6 Homs model of Inactivation kinetics for silver nanoparticle against E. coli

Table 3. Bacterial Inactivation Kinetics of Silver Nanoparticle

Inactivation models and parameters		Concentration of Silver nanoparticle (ppm)				
		0.1	0.2	0.3	0.4	0.5
Chick	k (min^{-1})	0.001	0.002	0.003	0.005	0.007
	R^2	0.946	0.968	0.932	0.958	0.911
Chick-Watson	$k^{'}$ (min^{-1})	0.006	0.013	0.016	0.024	0.037
	C (mg/L)	0.160	0.163	0.166	0.168	0.171
	n	0.987	0.989	0.992	0.995	0.999
	R^2	0.946	0.968	0.932	0.958	0.911
Homs	$k^{'}$ (min^{-1})	0.012	0.012	0.039	0.032	0.032
	C (mg/L)	0.181	0.183	0.186	0.175	0.177
	m	0.81	0.89	0.79	1.278	1.282
	n	0.982	0.983	0.986	0.991	0.995
	R^2	**0.965**	**0.971**	**0.956**	**0.975**	**0.981**

Homs inactivation parameter values of n were observed in the range from 0.307 (0.1 ppm) to 0.753 (0.5 ppm), indicating that the dosage was important for increased inactivation. The order reaction rate constant (m) values were observed in the range from 1.02 (0.1 ppm) to 1.3 (0.5 ppm), representing the shoulder curve of inactivation. The shoulder curve of inactivation may be due to the agglomeration of E. coli cells (Brahmi and Hassen 2011) and the greater m values (m>1) showed an increased inactivation rate(Gangadharan D et al 2010). The formation of the shoulder region was due to the increase of damaged E.coli cells rather than instant death which indicates the tailing curve.

CONCLUSION

The studies concluded that the rapid synthesis of silver nanoparticle was achieved using silver resistant P. putida. The optimum threshold concentration of silver nitrate for P. putida was found to be 1 mM. The UV spectroscopic peaks for the synthesized silver naoparticle were measured at 420 nm, the Surface Plasmon Resonance region confirmed a characteristic peak for metal ions. The cloud point extraction was used to extract silver nanoparticle successfully and the characterization studies confirmed the spherical morphology with the average size of 57 nm. The antibacterial studies using different concentrations of silver nanoparticle against E. coli was observed with lagging growth and complete growth inhibition was achieved at 0.5 ppm concentration. The morphological denaturation and cytoplasmic leakage of treated E. coli were examined and the shoulder curve of inhibition was inferred, well explained by Homs model of inactivation. These results revealed the ecofriendly preparation of silver nanoparticle and their potential antibacterial activity could be beneficial in the preparation of disinfectant filters for wastewater treatment and in the preparation of antibacterial materials in medical fields.

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