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Biocidal Nature of Combined Treatment of Ag-nanoparticle and Ultrasonic Irradiation in *Escherichia coli dh5α*

Dhermendra K. Tiwari and J. Behari

School of Environmental sciences, Jawaharlal Nehru University, New Delhi, 110067- India

Abstract: Ultrasound cause bacterial cells damage and Ag-nanoparticle is a very good antibacterial agent against gram (-) bacteria. We have studied synergistic effect of Ag-nanoparticle/ultrasound in *E.coli*. When the *E.coli* cells were treated with both Ag-nanoparticle and ultrasound, the Biocidal effect was more pronounced compared to the single treatment. This is also supported by Transmission electron microscopy (TEM) study. It was found that the effect is visible even in short time treatment (5minute) of these two. It is suggested that the synergistic treatment cause more damage in bacterial population than single treatment (either Ag-nanoparticle treatment or ultrasound treatment). It is suggested that this technique can be used for waste water treatment.

Key words: Nanoparticle · Ultrasound · Synergistic effect · Colony forming unit · Acoustic energy

INTRODUCTION

Among various microbial inactivation methods ultrasound is known one of them since ancient times [1]. Several investigations have been carried out to study the inactivation effect of ultrasound [2, 3] and ultrasound combined with other agents [4,5]. It has been reported that the simultaneous heat and ultrasound treatment (thermo ultrasonication) has a higher lethal effect than the heat treatment at the same temperature [6]. Ultrasound is able to inactivate bacteria and deagglomerate bacterial clusters through a number of physical, mechanical and chemical effects arising from acoustic cavitation. Cavitation bubbles formation is the cause of the production of energy to mechanically weaken or disrupt bacteria or biological cells via Shear forces (induced by micro streaming occurs within bacterial cells), Chemical attack (due to the formation of radicals; ●OH and ●H during cavitation) and Forces (due to surface resonance of the bacterial cell are induced by cavitation) [7]. On the basis of these facts, some recent studies have dealt with the use of low frequency ultrasound (in the range 20-40 kHz) alongside ozone [8], ultraviolet irradiation [9], hydrodynamic cavitation [10], electrolysis [11], chlorination [12-14] and heterogeneous catalysts (i.e. activated carbon, ceramic, zinc and titanium dioxide) [15,16] and reported that enhanced disinfection efficiencies could be achieved with the combined treatments.

Ag-nanoparticle has been tested in various field of biological science Viz. drug delivery, wound treatment, binding with HIV gp-120 protein [17], in water treatment and an antibacterial compound against both Gram (+) and Gram (-) bacteria [18-24]. Most of the bacteria have yet developed resistance to antibiotics. Viewing all the above facts, it is future need to develop a substitute for antibiotics [20]. Ag-nanoparticles are attractive as these are non-toxic to human body at low concentration and having broad-spectrum antibacterial nature. Ag-nanoparticle inhibits the bacterial growth at very low concentration than antibiotics and as of now no side effects are reported [21]. Ultrasound increases transport of small molecules in a liquid solution by increasing the convection in stagnant or relatively slow moving fluid [25-28] and also increase the DNA transfer in *E.coli* [29]. This study supports that the ultrasound can thus facilitate the entry of Ag-nanoparticles to bacterial cells and shows enhanced antibacterial properties. Depending on the strength and frequency of waves, cell wall structure and sonication environment, the impact of ultrasound would be different. On the basis of some positive findings of combined treatment with ultrasound (as above mentioned), we have studied the combined effect of ultrasound/Ag-nanoparticle because Ag-nanoparticles are highly reactive species because of the large surface area and very strong antibacterial agent. We have studied the biocidal effect of Ag-nanoparticle against *E.coli* bacteria in presence of short and long exposure of ultrasound (35 KHz and 135 KHz).

Corresponding Author: J. Behari, School of Environmental sciences, Jawaharlal Nehru University, New Delhi, 110067- India

MATERIAL AND METHOD

Chemicals and Media: All chemicals were obtained from Sigma-Aldrich Co., India and were analytical-grade reagents unless otherwise stated. Luria–Bertani (LB) medium was used for the cultivation of bacteria. Ag-nanoparticle was used and was obtained locally.

Organism Preparation: *E. coli DH5* strain were grown overnight in LB at 37±2°C. Bacterial cells were centrifuged at 6000 rpm for 15 minute; washed cell pellets were resuspended in PBS buffer and optical density (OD) was adjusted to 0.1, at 595 nm.

Ultrasonic Treatment of Bacterial Culture for Various Time Interval and Different Frequencies: Active bacterial culture of 10 ml (0.1 O.D.) was exposed in ultrasonic irradiation at 35 KHz and 135 KHz for 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 minute. Treated culture was inoculated in fresh LB tubes and incubated for 24 hrs in BOD shaker. O.D. was measured after 24 hrs incubation.

Combined Effect of Ag-Nanoparticle /Ultrasonic Irradiation: 1.0 ml of bacterial sample was treated with 1.0 ml of Ag-nanoparticle solution with final concentration of 15, 25, 35, 45, 55, 65, 75 µg/ml of homogenized Ag-nanoparticle and at the same time, all these samples were also exposed for 0, 5 and 15 minute at 35 KHz and 135 KHz. All treated sample were inoculated in 10 ml LB broth and incubated for 24 hrs in BOD shaker at 140 rpm. O.D. was measured by UV-Visible spectrophotometer. All assays were carried out in duplicates in an effort to eliminate any possible error.

TEM Image of Ag-Nanoparticle/Ultrasonic Irradiated Cells: To examine the Ultrasonic effect and Ag-nanoparticle interaction with bacterial strains, cells were grown in liquid LB medium at 35±2°C for 24 hrs. Cells were harvested by centrifugation at 6000rpm (15 minute) and mixed in PBS. Bacterial cells were treated with ultrasound and followed by Ag-nanoparticle (2 hrs). Nanoparticles interaction with bacterial cells was characterized by Transmission Electron Microscopy (TEM). The cells treated only with ultrasonic irradiation were also studied by TEM. The effect of treatment on the bacteria was monitored by depositing 10 µl of each sample on carbon-coated copper TEM grids followed by air-drying. TEM image of the ultrasound/Ag-nanoparticle treated *E. coli* cells were analyzed by a Hitachi FEI. Bacterial cells were treated with only ultrasound or nanoparticle used as a control.

RESULT

Bacterial Treatment with Ultrasonic Irradiation for Different Time Interval: Bacterial cell were treated with 35 KHz & 135 KHz ultrasound show continuous decrease in O.D (figure-1). The cells treated with different time interval from 5 to 60 minute show continuous decrease in cell count. When the cells treated at 135 KHz, colony count was less as compare to 35 KHz. When the cells were treated with 135 KHz for 25 minute, ~50% decrease in cells count was appeared but it was only ~25% with 35 Khz. The time at which all cells lost viability was 60 minute at 35 KHz and 45 minute at 135 KHz. It can be said that the higher frequency of the ultrasound is more effective at different time intervals. It is suggested that the cell lost viability due to membrane damage by acoustic energy produced by ultrasound. As exposure time of ultrasound increases the cell lost the control over transport mechanism and after long time exposure, most of the cells may loose viability. When the cells lost control over membrane transport, membrane potential will be affected and ion balance between cytoplasm and extracellular space will be affected, ultimately cells lost control over all metabolic activities.

Growth Pattern for Combined Treatment of Nanoparticle and Ultrasonic Irradiation: From figure-2, it is evident that the O.D. of the growing culture decreases when the cells were treated with both ultrasound (35 KHz & 135 KHz) and Ag-nanoparticle. When the treatment time of ultrasound was increased, the viability loses of bacterial cells increased. The synergistic effect was higher at 135 KHz as compared to 35 KHz ultrasound frequency (when all the experimental conditions were same in both). When the cells were treated with 135 KHz for 15minute in 25µg/ml Ag-nanoparticles solution, 50% viability loses appeared but it was 40% with 35KHz (when all the experimental conditions are same). It can be suggested that the higher frequency of ultrasound is more potent and able to facilitate the entry of nanoparticle with short time exposure. It has been reported that the ultrasound permits the transport of molecule from LB medium to bacterial cells. Ultrasound may facilitate the entry of Ag-nanoparticles present in growing media and shows enhanced antibacterial properties. Ultrasound increases the convection of liquid by at least two mechanisms. The first is acoustic streaming flow in which the momentum from propagating sound waves is directly transferred to the liquid, causing the liquid to flow in the direction of the sound propagation. Thus any amount of ultrasound in liquid produces additional convecting transport from acousting streaming. The second and more notable

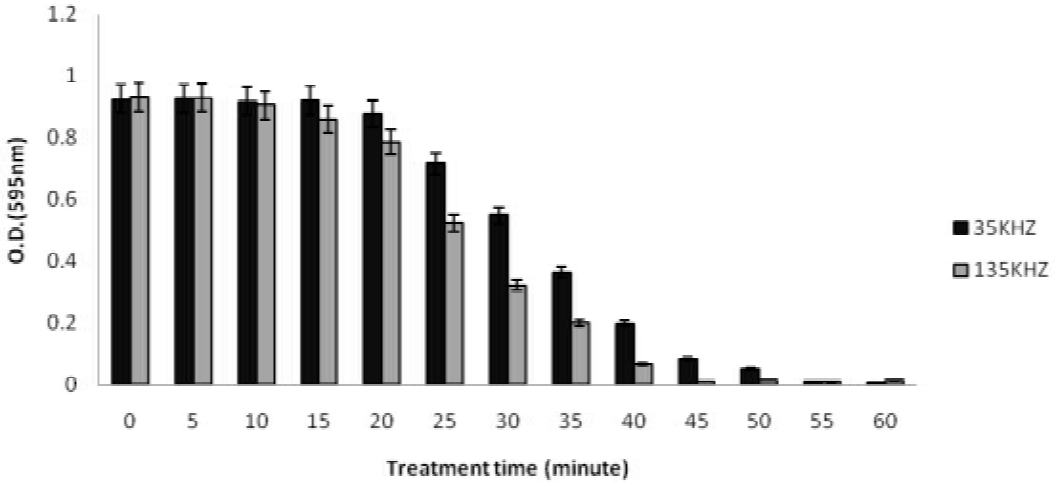


Fig. 1: Bacterial cells were exposed in ultrasound at 135 KHz & 35 KHz. Cells were exposed in two different frequencies (135 KHz & 35 KHz) for various time periods as mentioned in X-axis and O.D. was measured after 24 hrs incubation in rotatory shaker at 37°C.

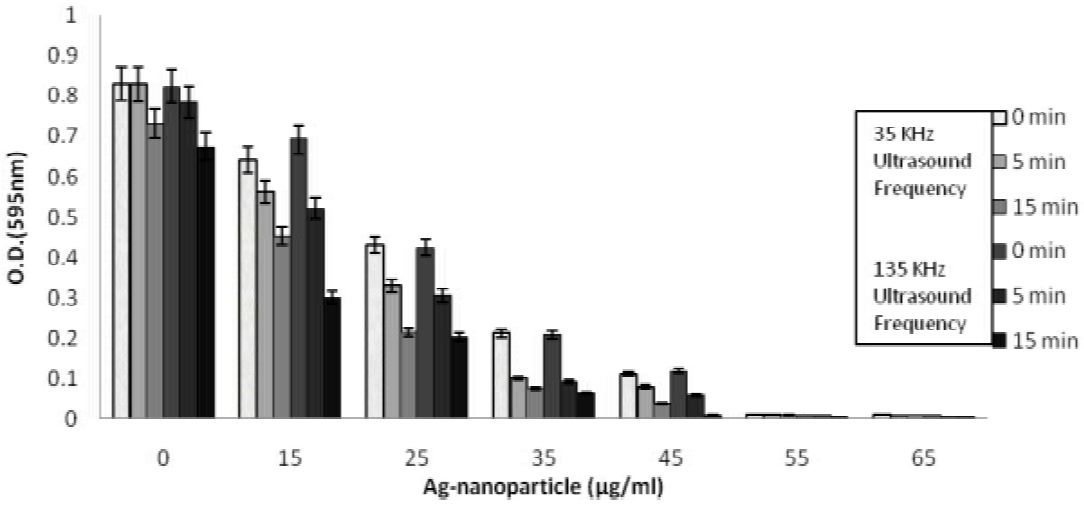


Fig. 2: Synergistic treatment of bacterial cell with Ag-nanoparticle/Ultasound. E.coli cells were treated with various concentration of Ag-nanoparticle (as on X-axis) and same time it's also treated with ultrasound (35 KHz & 135 KHz) for 5 and 15 minute. Sample treated with ultrasound only used as control. Figure shows that the synergistic effect is much higher than the culture treated only with ultrasound. It indicates that the ultrasound facilitate entry of nanoparticle inside the bacterial cells.

example of enhancing convection is known as microstreaming and is produced by cavitating gas bubbles to expand and shrink, which in turn creates shear flow around the oscillating bubbles.

TEM Image of Ultrasound/Nanoparticle Treated Cells: Figure-3 show TEM image of ultrasonic/nanoparticle treated cells. Interaction between gram-negative *E. coli* with silver nanoparticles is illustrated in the transmission electron microphotographs and the damage occurred by long time exposure of ultrasound shows in figure-3. In the short incubation of bacterial cells with Ag-nanoparticle, particles appear on bacterial surface but when the cells treated with both ultrasound/nanoparticle, the nanoparticles enter inside the cells and show pronounced effect. It is expected that the particle bind with the region, rich in negatively charged functional groups either in protein or in to DNA. Long time

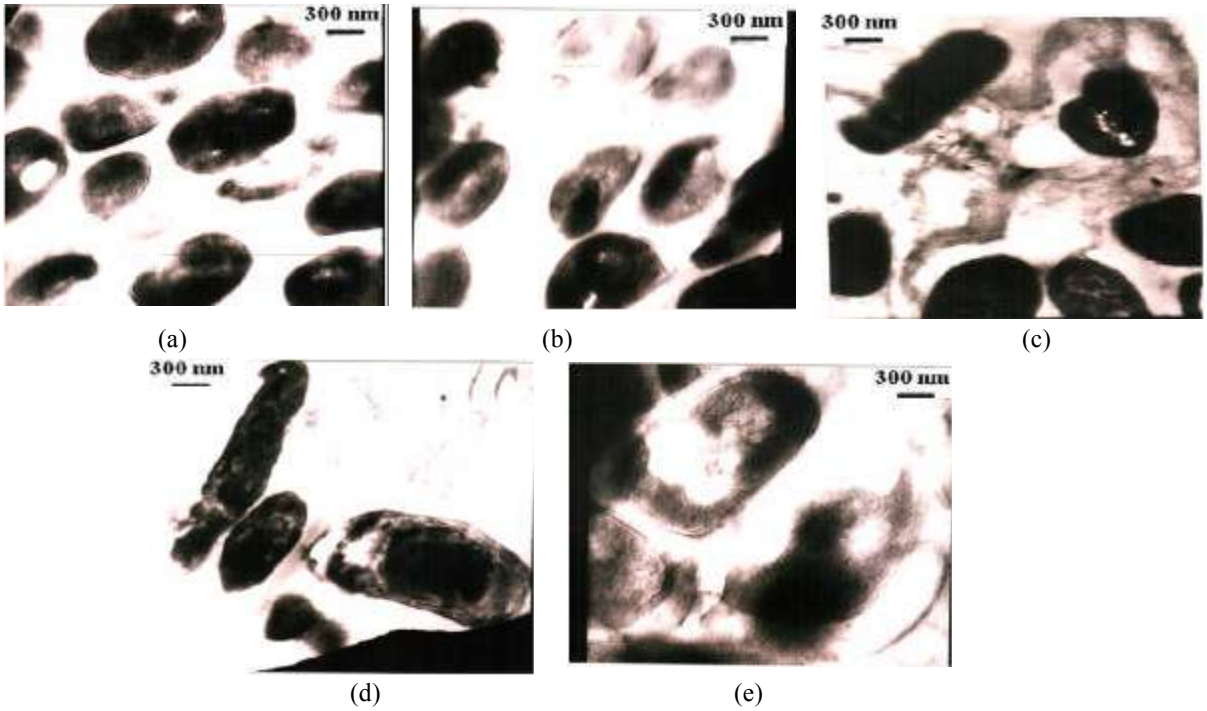


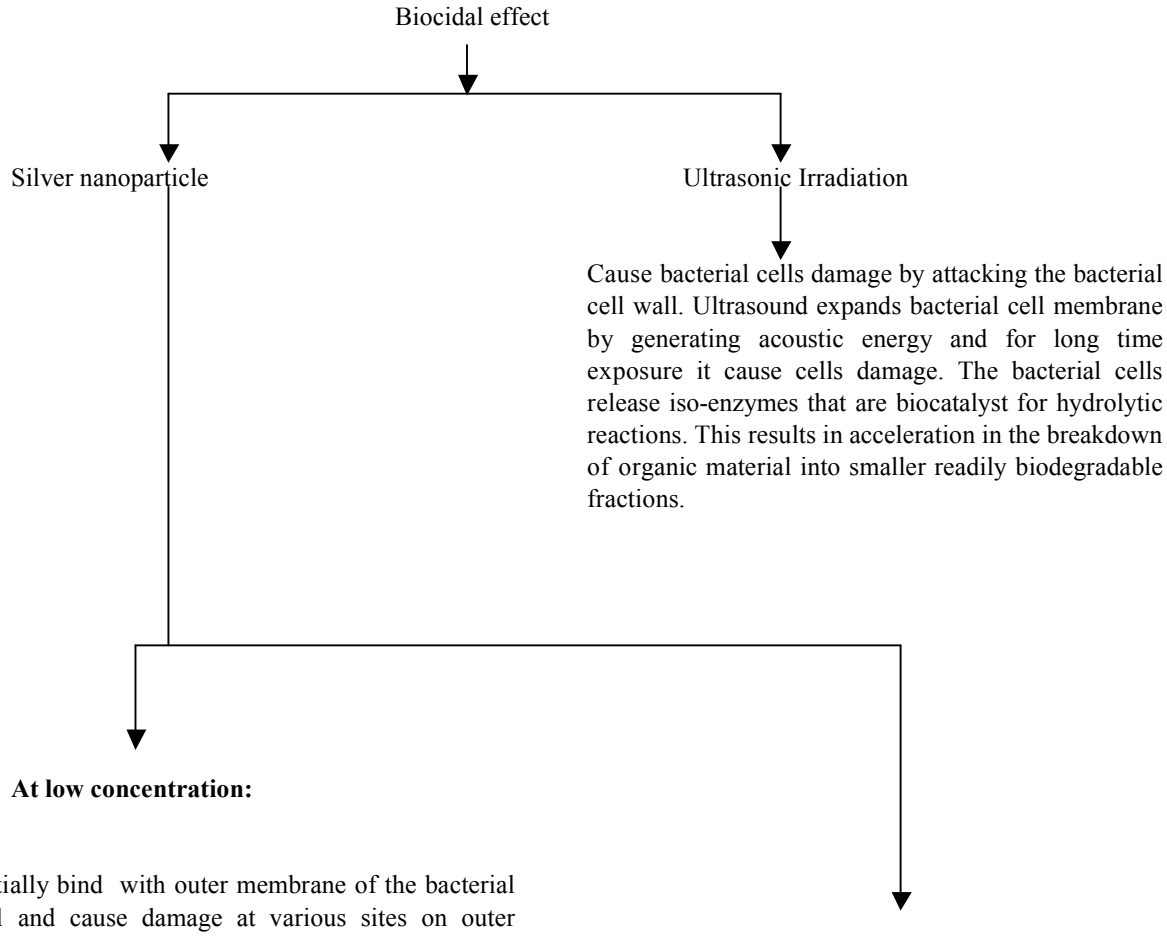
Fig. 3: TEM image of the bacterial cells treated with Ag-nanoparticle/Ultrasonic irradiation.
(A). Cells without treatment (B). Cell treated with 35 KHz ultrasonic irradiation (C). Cells treated with 135 KHz ultrasonic irradiation D. Cells Treated with Ag-nanoparticle only (E). Cells treated with both Ultrasound /Nanoparticle

incubation without ultrasonic treatment shows that the nanoparticles enter inside the bacterial cells, if the cells treated combined with Ag-nanoparticle and ultrasound (for short time period), it shows presence of nanoparticle inside the cells. It can be suggested that the biocidal effect of nanoparticle enhances, when the treatment coupled with short time ultrasound exposure. These results indicate that the short exposure of ultrasound facilitates the entry of Ag-nanoparticle inside the cells. It is expected that the nanoparticles anchor with the cell surface at several sites and cause damages at various sites in the membrane, which could result in cell lysis. It has been predicted that the nanoparticle bind with sulphur rich region on the cell wall of bacteria. When the particles enter inside the cells, it might be bind with negatively charged group containing proteins and nucleic acids.

CONCLUSION

The present study shows the synergistic effect of Ag-nano/Ultrasound. Ultrasound cause cells destruction (at 35 Khz and 135 Khz) but within a certain time limit it may not cause any damage in bacterial cells. In this study it was found that the nanoparticles treated with short time exposure with ultrasound show increased antibacterial effect but this time was not enough to kill the bacterial cells with ultrasound only. It indicates that the ultrasound facilitates the entry of Ag-nanoparticle inside the bacterial cells and the antibacterial effect was enhanced with same concentration of nanoparticle in presence of ultrasound. This study can be the bases of water treatment in a fairly large sample.

Anticipated biocidal mechanism of ultrasound and silver nanoparticle in bacterial cells can be summarized thus



At low concentration:

1. Initially bind with outer membrane of the bacterial cell and cause damage at various sites on outer membrane.
2. Bind with sulfhydril group of bacterial Glycoprotein present in bacterial outer membrane.

    a. It affects the expression of membrane protein and the protein involved in membrane transport.
    b. It changes the native structure of protein, Which may affect the cell membrane integrity.
    c. The binding of Ag-nanopartcle cause the destabilization of outer membrane structure, collapse plasma membrane potential and deplete the intracellular ATP level.

At high concentration:

At high concentration level nanoparticle may enter inside the cell through porins present on membrane. Particle will bind with negatively charged cytoplasmic components (nucleic acid and protein). No any kind of nucleic acid damages found in previous study but in some studies, it is observed that the expression of some cellular and membrane proteins increases.

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