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Research Article

Antimicrobial Property of Zinc Oxide Nanoparticles on *Escherichia coli* and *Listeria monocytogenes* – A Comparative Study

A. Kalaiselvi[1], C. Ramalingam[1,*], G. Madhumitha[2,*].

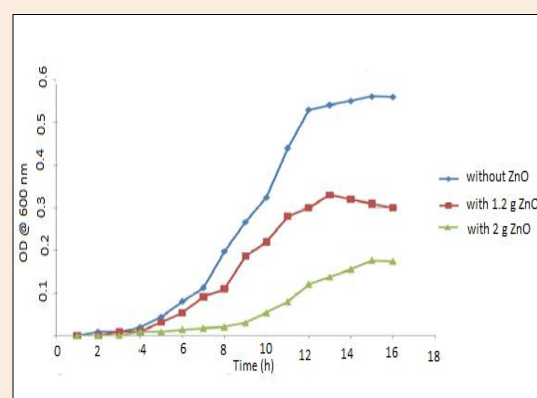
[1]Food Analysis and Instrumentation Lab, School of Biosciences and Technology, VIT University, Vellore, India
[2]Organic Chemistry Lab, School of Advance Sciences, VIT University, Vellore, India.

Abstract

ZnO nanoparticles are inorganic nanoparticles with effective antibacterial, antifungal, anti corrosive, catalytic and UV filtering properties. The ZnO nanoparticles were prepared through precipitation process and the characterization was done using XRD and AFM. Then using the standard microbial method the antimicrobial activity of ZnO nanoparticles at different concentrations was tested on *Escherichia coli* culture. Both ZnO and E.*coli* were placed on nutrient agar plates and incubated for 18-24 h. The ZnO nanoparticles were found to be bactericidal, different zones of inhibition were created for different concentrations. However ZnO suspensions in lower concentration range (0.01-1 mM) seemed to exhibit less antimicrobial activity. The presence of less zinc ions made zinc act as a nutrient. By varying the concentration the minimum inhibitory concentration for ZnO nanoparticles were calculated. Lower MIC corresponds to higher effectiveness. The minimum inhibitory concentration (MIC) for *E.coli* was found to be 20 mg/mL which gives a zone of inhibition of 8 mm diameter. The minimum inhibitory concentration for *Listeria monocytogenes* was found to be 30 mg/mL of ZnO nanoparticles.

Keywords: ZnO Nanoparticles, X-Ray Diffraction, AFM (Atomic Force Microscopy), Antimicrobial Activity.

It is observed that the zone of inhibition is 19 mm for *E.coli* at 50 mg/mL of ZnO and 20 mm for *Listeria monocytogenes* at 100 mg/mL of ZnO. Also the growth curve study was for the activity of bacteria with and without the presence of ZnO nanoparticles.



***Correspondence**
Dr.C.Ramalingam & Dr. G. Madhumitha
Email:cramalingam@vit.ac.in; madhumitha.g@vit.ac.in

1. Introduction

Nanoparticles with sizes in the 1-100 nm range, comparable to the size range of biological structures are attractive material for the manipulation, sensing, and detection of biological systems [1]. Nanophasic and nanostructured particles are attracting a great deal of attention because of their potential for achieving specific process and selectivity, especially in biological and pharmaceutical applications [2]. The ZnO nanoparticles can act as effective antimicrobial agents [3]. Its antimicrobial efficacy has been tested against *Listeria monocytogenes, Salmonella enteritidis* and *Escherichia coli*. This study gave green light to the use of ZnO nanoparticles in food systems. Some studies showed it can have inhibitory effect on human cells. According to these studies when reduced to nanoscale realm many benign materials develop toxicity. However developing selective toxicity to biological systems and controlling it by nanoparticles design it could lead to biomedical and antibacterial applications.

The particle size has an effective role to play in the antimicrobial efficacy of ZnO nanoparticles. Among various methods the particle size obtained through dissolving zinc acetate in 2-propanol having 2-mercaptanol as the

stabilizing method is much less duration compared to precipitation method [4]. The TEM studies showed that thiol capped particles are well separated compared to uncapped ZnO. Other methods for preparation include electrochemical synthesis via a metal-ligand-coordinated vesicle phase. The structure was determined through scanning electron microscope and energy dispersive x-ray spectroscopy which showed monodispersed nanoparticles with different average diameters.

Zinc oxide quantum dots are nanoparticles of purified ZnO. These were evaluated for their antimicrobial activity against pathogens. The quantum dots were utilized as a powder bound in polystyrene film or suspended in polyvinylpyrrolidone. Bacterial cultures were inoculated into culture media or liquid egg white. The ZnO-PVP (3.2 mg ZnO/mL) treatment resulted in 5.3 log reduction of *L. monocytogenes* and 6.0 log reduction of *E. coli* O157:H7 in growth media after 48 h incubation, as compared to the controls. *Listeria* cells in the LEW control increased from 3.8 to 7.2 log CFU/mL during 8 h incubation, while the cells in the samples treated with 1.12 and 0.28 mg ZnO/mL were reduced to 1.4 and 3.0 log CFU/mL, respectively. After 8 h incubation, the cell populations of Salmonella in LEW in the presence of 1.12 and 0.28 mg ZnO/mL were reduced by 6.1 and 4.1 log CFU/mL over that of controls, respectively. ZnO powder and ZnO-PVP showed significant antimicrobial activities against all 3 pathogens in growth media and LEW. ZnO-PVP coating had less inhibitory effect than the direct addition of ZnO-PVP. No antimicrobial activities of ZnO-PS film were observed. The inhibitory efficacy of quantum dots was found to be concentration dependent and also related to the type of application [5].

Summing all the papers together we come to the conclusion that despite of many methods, preparation of ZnO nanoparticles by chemical method is the simplest. All the methods give ZnO nanoparticles in the size range of 10-50 nm. The smaller the particles size the better antibacterial activity is given.

The concentration of zinc ions in the solution has a major role to play. The obtained nanoparticles if not present in sufficient concentration they might act as a nutrient [6]. If a stabilizing agent is used while preparing nanoparticles they cap nanoparticles which help to maintain its size stable compared to non-capped counterparts. Capped nanoparticles don't show any signs of agglomeration [7]. Moving further we also noticed that most of the papers centered on antibacterial property of ZnO were published in or after 2010 shows it is a recent discovery for awaiting further experiments to be done on them. Regarding antibacterial property, ZnO is used in dental composites which itself proves that it is non-toxic to human body. It is in particular effective against *Listeria monocytogenes, Escherichia coli* and *Staphylococcus aureus* showing its worth in food packaging industry.

2. Experiment

2.1 Materials and Methods:

The ZnO nanoparticles were synthesized using zinc nitrate (0.1M) and sodium hydroxide (0.2M). Hot air oven and heat furnace were used as well in the precipitation process. Double distilled water was used for washing. Hydrogen peroxide was used to make a translucent solution. For antibacterial experiments *Escherichia coli*, gram negative bacteria, was chosen by us. The nutrient broth and Mueller-Hinton agar was chosen for culturing *E.coli*. The Petriplates, beaker and other glassware used were properly sterilized. Another culture used for testing antibacterial properties of ZnO nanoparticles was *Listeria monocytogenes*. Heart brain infusion broth and heart brain infusion agar was used for culturing.

2.2 Synthesis of ZnO Nanoparticles:

Zinc nitrate (0.1M) and sodium hydroxide (0.2M) were constantly stirred at room temperature for two hours. After two hours a white precipitate was formed which was washed with double distilled water to remove all the ions. The obtained sample was then centrifuged at 3000 rpm for 5 min. Then the supernatant was discarded and the precipitate

was collected. Then 1 mol of hydrogen peroxide is added at 75° C to make a translucent solution for 1h. The solution was then dried in hot air oven at 60° C for 3 h. It was further calcined at 350°C for 6 h to form ZnO crystals.

2.3 Characterization of ZnO Nanoparticles

2.3.1 X-Ray Diffraction

X-Ray diffraction patterns were recorded from 20° to 90° with an analytical system diffractometer (Model: DY-1656) using Cu Kα (λ=1.542A°) with an accelerating voltage of 40KV. Data were collected with a counting rate of 1°/min. The Kα doublets were well resolved. From XRD, the crystallite size can be found out by using the Scherrer's formula:

$$P = k \lambda / \beta \cos\theta$$

Where,

P – Crystallite size
λ – Wavelength (1.54A°)
β - Full maxima half width,
θ- Diffraction angle
k- Scherrer's constant

2.3.2 Atomic Force Microscopy

The powder sample of ZnO nanoparticles was not soluble in most acids including sulphuric acid. It is only soluble in diluted solution of CH_3 COOH. 1 mg of ZnO was dissolved in 2 % dilute acetic acid. A thin layer of this powder was spread on glass slide using another glass slide at 45° angle. The motive was to form a very thin almost invisible layer which could be detected easily for the particle characteristics.

2.4 Analysis of Antibacterial properties

We selected two organisms for our study. The organisms were identified by Microbial Identification System as well as Biomedical tests (NIO, Kochi).

2.4.1 Antibacterial tests on Escherichia coli

The first test was done on *E.coli*. All the materials were sterilized in an autoclave before starting the experiment. In the liquid culture the density of bacterial cells was estimated using optical density tests at 600 nm wavelength and was kept within the range of 0.8-1.0 nm, which is considered to be the most ideal optical density of cells. The antibacterial property of ZnO was measured in terms of zone of inhibition and minimum inhibitory concentration (MIC) was calculated.

The petri plates used in the tests were prepared using a Mueller-Hinton agar medium. In the preliminary test bacteria were sprayed evenly on top of the plates using a sterile glass rod. After allowing the bacteria to dry (within 5–10 min) test solutions of ZnO nanoparticles of two different amounts 1 mg and 2 mg were dropped within the wells of 5mm diameter. Also in the 3rd well control was added. After 18 hours of incubation, the petri plate was observed for any zone of inhibition in all the wells.

2.4.2. Antibacterial tests on Listeria monocytogenes

Listeria monocytogenes is a gram positive, facultative anaerobe and intracellular bacterium. It is the causative agent of listeriosis. It is one of the most virulent foods borne pathogen. All the materials used were sterilized in an autoclave before experiment. Brain heart infusion (BHI) broth was used as source for culturing *Listeria monocytogenes* at room

temperature on a rotary platform. The antibacterial activity of ZnO nanoparticles was measured by well diffusion method in terms of zone of inhibition and minimum inhibitory concentration (MIC). The petri plates used in the tests were prepared using a Brain heart infusion agar medium. In this test the bacteria were sprayed evenly on top of the plates using a sterile glass rod. After allowing the bacteria to dry (within 5−10 min) test solutions of ZnO nanoparticles of various concentrations were dropped within the wells of 5 mm diameter. The petri plates were observed for any zone of inhibition after 24 h of incubation at room temperature.

2.5 Bacterial growth study

A comparative study is made between normal bacterial growth and nanoparticles (ZnO) induced bacterial growth. All the materials to be used were sterilized before the experiment.

2.5.1 For E.coli growth study

Three side arm flasks were taken. Each flask was filled with 40 mL of nutrient broth and inoculated with 100 µL of *E.coli* culture. At the 0th h of growth one of the flasks were added with 0.8 g of ZnO nanoparticles as the MIC (minimum inhibitory concentration) for *E.coli* is 20 mg/mL. 2nd flask was added with 2 g of the nanoparticle. 3rd flask was left as such (control), At regular intervals of 1 h the OD value was measured at 600 nm using colorimeter till 16 h. The OD values were tabulated and the graph was plotted. All the growth curves were compared and studied.

2.5.2 For Listeria monocytogenes growth study:

The same procedure is followed as it was done for *E.coli*. Three side arm flasks were taken. Each flask was filled with 40 mL of Brain Heart Infusion broth and inoculated with 100 µL of *L. monocytogenes* culture. At the 0th h of growth one of the flasks were added with 1.2 g of ZnO nanoparticles as the MIC (minimum inhibitory concentration) for E.coli is 30 mg/mL. 2nd flask was added with 2 g of the nanoparticle. 3rd flask was left as such (control). At regular intervals of 1 h the OD value was measured at 600 nm using colorimeter till 16 h. The OD values were tabulated and the graph was plotted. All the growth curves were compared and studied.

3. Results and Discussions:

Characterization of ZnO nanoparticles

3.1 XRD analysis

XRD analysis of ZnO nanoparticles yielded a graph which was matched with the JCPDS (joint community on power diffraction standards) using MATCH software. The XRD analyzed graph matched exactly with the standard showing the quality of the product as excellent. Using information from XRD analysis the ZnO nanoparticles size was calculated roughly using Scherrer's formula.
Initially wavelength (λ) was calculated using Bragg's equation $n\lambda = 2d \sin\theta$
Scherrer's formula: $P = k \lambda / \beta \cos\theta$

Particle sizes calculated by Scherrer's formula ranged from 10 nm-40 nm. The average particle size was around 20 nm.

3.2 AFM (Atomic force microscopy analysis)

The AFM analysis yielded the particle size of ZnO nanocrystals. The size ranged between 20-50 nm which was coinciding with the particle sizes calculated using Scherrer's formula. The average particle size was around 34.5 nm.

3.3 Antimicrobial activity of ZnO nanoparticles

3.3.1 Antibacterial activity on E.coli

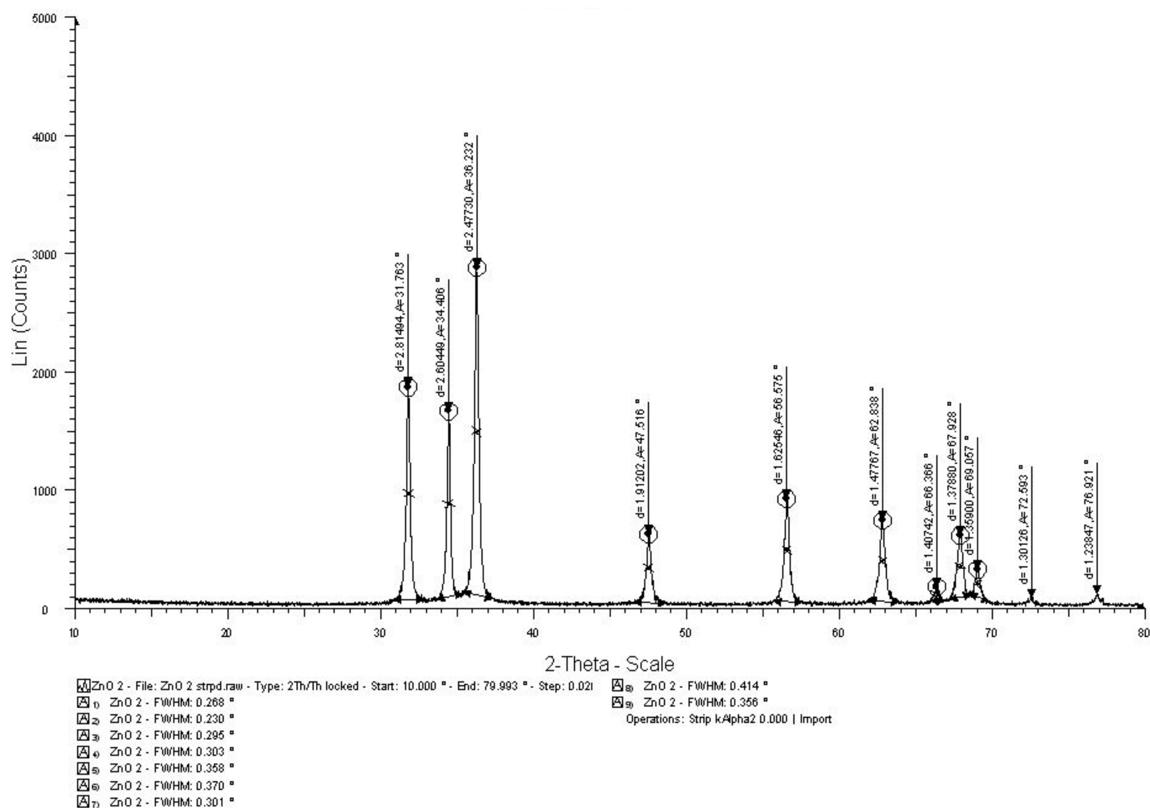


Figure 1 Graph yield by XRD analysis

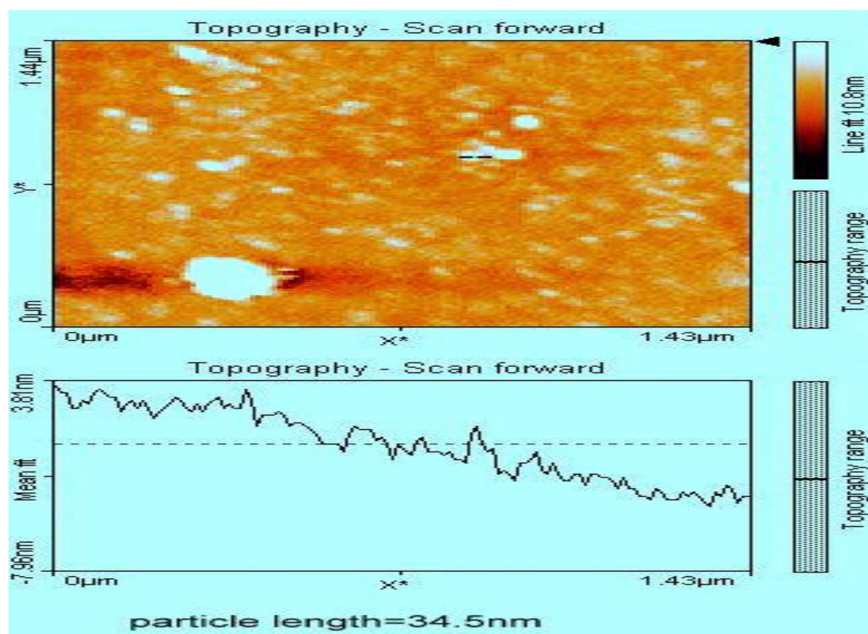


Figure 2 AFM image showing ZnO nanoparticles size

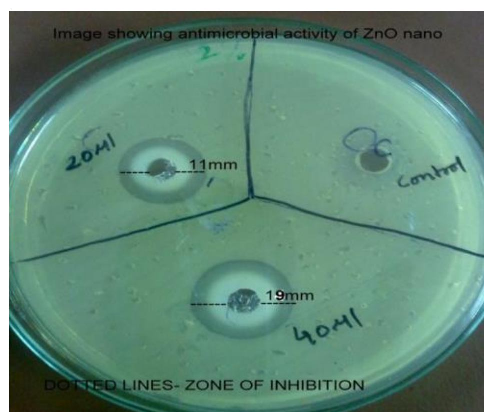


Figure 4 Zone of inhibition found in the preliminary test on E.coli

In the preliminary test bacteria were sprayed evenly on top of the plates using a sterile glass rod. Test solutions of ZnO nanoparticles of two different amounts 1 mg and 2 mg were added within the wells of 5 mm diameter. Also a control was kept with 2 % acetic acid (v/v). The well with 1 mg of ZnO nanoparticles yielded 11 mm of zone of inhibition. The well with 2 mg of ZnO nanoparticles yielded 19 mm of zone of inhibition. It showed that control did not have any effect on the bacterial colonies.

The petri plates used in this tests were prepared using a Mueller-Hinton agar medium. In this test bacteria were sprayed evenly on top of the plates using a sterile glass rod. Test solutions of ZnO nanoparticles of various concentrations were dropped within the wells of 5 mm diameter. After 18 h of incubation the petri plates were observed. The minimum inhibitory concentration was found to be 20 mg/mL which gave a zone of inhibition of 8mm diameter. As the concentration of ZnO nanoparticles decreases the zone of inhibition also decreases.

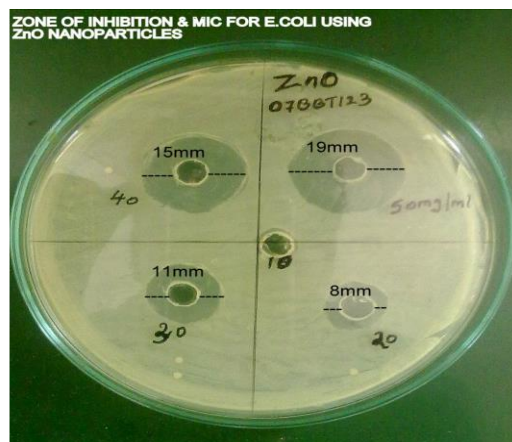


Figure 4 minimum inhibitory concentrations for E.coli

3.3.2 Antibacterial activity of ZnO nanoparticles on Listeria monocytogenes

Same procedure was followed as it was done for *L.monocytogenes* except for the changes that the broth and agar used were of Brain Heart Infusion. After 24 h of incubation the petri plates were observed for the zones of inhibition and were tabulated. The minimum inhibitory concentration was found to be 30 mg/ml of ZnO nanoparticles. *Listeria monocytogenes* is a gram positive bacteria and *E.coli* is gram negative. It is observed that the zone of inhibition is 19 mm for *E.coli* at 50 mg/mL of ZnO and 20 mm for *Listeria monocytogenes* at 100 mg/mL of ZnO. For almost same

zone of inhibition it required double the concentration of ZnO for *Listeria monocytogenes* when compared to *E.coli.* Image showing zones of inhibition and minimum inhibitory concentration (MIC) for *Listeria monocytogenes*

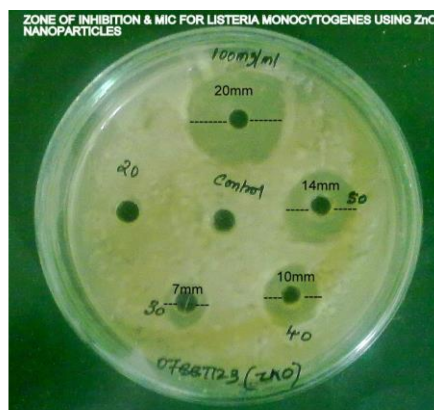


Figure 5 minimum inhibitory concentrations for Listeria monocytogenes

5.4 Bacterial growth study

A comparative study is made between normal bacterial growth and ZnO nanoparticles induced bacterial growth.

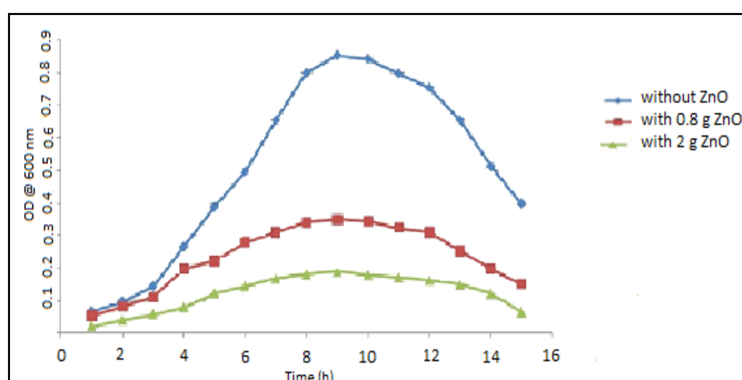


Figure 6 Growth study of *E.coli*

A comparative graph showing the growth curves of *E.coli* cultures with and without ZnO nanoparticles at 600 nm OD. Graph was plotted with time on Y-axis and OD values on X-axis using Microsoft office Excel. The growth curve study reveals that the culture containing ZnO nanoparticle has less growth when compared with that of normal one. Growth is minimum in the culture with 2 g of nanoparticles.

A comparative graph showing the growth curves of *L.monocytogenes* cultures with and without ZnO nanoparticles.

The growth curves of *Listeria monocytogenes* reveals that the culture containing ZnO nanoparticles has less growth when compared with that of normal one. Culture with 2 g of ZnO has minimum growth.

Conclusion

We have demonstrated the synthesis of ZnO nanoparticles through precipitation process. The quantity acquired was sufficient for carrying out one batch of experimental procedures. The size and morphology of nanoparticles was desirable for carrying out antimicrobial experiments. We can say precipitation is a safe method for synthesis of ZnO

nanoparticles. Also the antimicrobial activity of ZnO nanoparticles was achieved for various concentrations. XRD analysis revealed that the sample synthesized was free of impurities. The XRD analyzed graph matched exactly with the standard showing the quality of the product as excellent. The crystal structure was found to be hexagonal. The average particle size was calculated through Scherrer's formula and was found to be 20 nm. The particle size analyzed by AFM (atomic force microscopy) was supported by XRD scherrer's formula. The size ranged between 20 to 50 nm which were coinciding with the particle sizes calculated using Scherrer's formula. The average particle size was around 34.5 nm. ZnO nanoparticles have enhanced antimicrobial activity as the concentration increases the zone of inhibition also increases. Large numbers of active oxygen species are generated from ZnO particles onto the surface of colony which kills bacteria more effectively. Due to the damage of cell membrane it leads to the leakage of genetic materials, minerals and proteins causing cell death. It is observed that the zone of inhibition is 19 mm for *E.coli* at 50 mg/mL of ZnO and 20 mm for *Listeria monocytogenes* at 100 mg/mL of ZnO. In the antimicrobial study of *E.coli* and *Listeria monocytogenes* ZnO nanoparticles have less effect on *Listeria* as it is gram positive bacteria which has a thick peptidoglycan layer. However the antimicrobial activity on *Listeria* can be increased by increasing the concentration of ZnO nanoparticles. The comparison study of growth curves further solidated the fact that ZnO nanoparticles have antibacterial properties. All the phases in the life cycle of bacteria were affected significantly in the presence of ZnO nanoparticles. In Future, ZnO nanoparticles can be impregnated in to the polymer which can be used in food packaging. Medical devices can be coated with these nanoparticles against microbes. These nanoparticles can be used in making cigarette filters which can filter significant amounts of HCN and H_2S. The filter is made by impregnating ZnO and iron oxide in to the charcoal. ZnO nanoparticles are antimicrobial which can be used as an antiseptic. Further it blocks UV rays and hence can be used in sunscreen lotions.

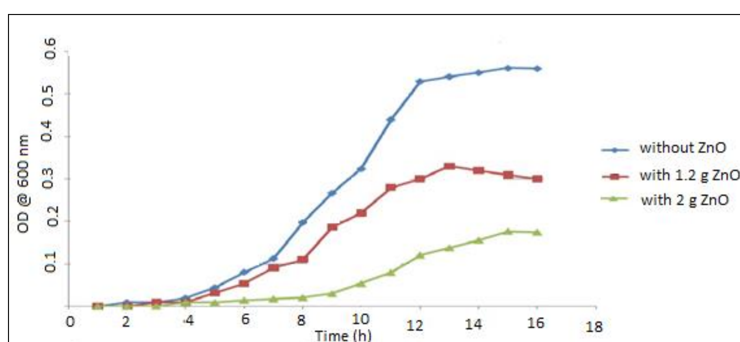


Figure 7 Growth study of Listeria monocytogenes

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