ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at www.jgpt.co.in

RESEARCH PAPER

Eco Friendly Synthesis, Characterization and Antibacterial Activity of ZnO Nanoparticles using *Bacillus Subtilis* against Multi-Drug Resistant Bacteria

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Abstract

Zinc oxide nanoparticles have received likely attention due to their immense uses. For such purpose, the improvement of innovative and biological methods is in significant request for raising these materials in industrial field. This document describes a unique way for the biosynthesis of ZnO nanoparticles using a*bacillus subtilis*. The morphology structure and stability of the synthesized ZnO nanoparticles were studied using XRD, FTIR, FE-SEM and TEM. The results showed that the synthesized nanoparticles are stable, hexagonal phase, spherical with extreme particles in size range within 7–19 nm in diameter and has antibacterial activity against MDR bacteria from different sources.

Keywords: *Eco friendly Synthesis*.

Introduction

The secrets pick up from natural surroundings have run to the expansion of biomimetic methods for the development of innovative non material's. Biological approaches designed for nanoparticle production using microorganisms, enzymes, and plants or plant extracts have been proposed as potential ecofriendly options to chemical and physical methods.

Zinc oxide nanoparticles (ZnO NPs) have gained vast acceptance in the scientific domain due to their unique and charming properties[1].In recent times, ZnO NPs has come in the scientific public eye, for its semiconducting properties, distinctive antibacterial, antifungal, wound curing and UV filtering properties, high catalytic and photochemical action. Interestingly researchers have used ZnO nanoparticles for the immobilization of beta galactosidase[2].

ZnO nanoparticles were made by several different procedures, such as the sol–gel techniques, wet chemical method, and green chemistry[3]a number of methods reported for synthesis of nanoparticles. Biological method is of great importance because, in the physical and chemical methods the usage of expensive chemicals as reducing and capping agents and toxic solvents along with the boring procedure control, as well as heating at high temperature at reduced pressure limits their biological uses[1].

To overcome these concerns, there is abundant request for biogenic Zinc oxide nanoparticles. The present research work describes the zinc oxide nanoparticles biosynthesis using reproducible bacteria, *bacillus subtilis*as eco-friendly reducing and capping agent. *Bacillus subtilis*is a gram positive, facultative anaerobe, nonpathogenic further most plentiful bacteria dispersed internationally in diverse soils.

Like most of the bacteria, *bacillus subtilis* have electro kinetic negative potential; which certainly attracts the cations and this work as a trigger for the biosynthesis of ZnO NPs?

Experimental

This method was done according to [4]with some modified. Pure culture of *bacillus subtitles* was inoculated into flask containing sterile 100 ml nutrient broth (cooled) under the aseptic condition of laminar air flow. 0.5 mm of Zinc Acetate dehydrates -Zn $(CH_3CO_2)_2$was added to the nutrient Broth media. Then it was incubated in shaker incubator at 37˚C for overnight for the growth of bacteria and then this mixture heating at 40°C for 3 hours. After this, the flask follow by cooling that allowed the nanoparticles to settle down.

The formation of nanoparticles was confirmed by observing the white color deposition at the bottom of flasks. After this we notes the mixture was separated the supernatant was removed then the centrifugation was done to pellet at 4000 rpm for 10 minutes washed with deionized water and centrifugation was done at 4000 rpm for 10 minutes and this was repeated a number of times .After every centrifugation the pellet was washed properly with deionized water. Finally the pellet was collected in a small plate and it was kept for drying in oven at 50°C till it was totally dry and ZnO nanoparticle was obtained in powdered form [4]

Characterization of ZNO Nanoparticles

X-ray Diffraction was performed using Powder X-ray Diffract meter (Shimadzu Xrd-6000-Jaban) supplied with CuKα radiation λ1.5405Å over a wide range of the Bragg angles θ ($10^o \leq 2\theta \leq 80^o$).For optical properties, UV absorbance spectra were measured by UV spectroscopy (Shimadzu -1800-Jaban).

The morphology and structure of nanoparticles were examined by field emission electron microscope (FE-SEM) analysis was performed on (type- S-1640 HITACHI company-Japan).The Transmission electron microscope type- JEOL 100CX II (TEM) 100kV –Al-Sharif University-IRAN image was recorded by dissolving the synthesized powder sample in ethanol and then placed a drop of ethanolic solution on the surface of copper grid. Characterization involved FTIR (IRAffinity-1, Shimadzu, Japan) analysis of the dried powder of synthesized ZnO NPs by scanning in the range 400–4000 cm^{-1} at are solution of $4cm^{-1}$.

Bacterial Isolates Used in the Study

Four multidrug resistance bacterial isolates were provided by different sources that belong to Gram negative and Gram-positive bacteria mention in Table (1).

The four isolates after obtained were confirmed by vitek 2 system for its identification and antibacterial sensitivity.

Table 1: Bacterial isolates

	Bacterial isolates	Source Obtained
1	Pseudomonas aeruginosa	Kufa university – college of science
2	Methicillin-resistant Staphylococcus aureus(MRSA)	Babylon university – college of medicine
3	Escherichia coli	Kufa university – college of science
4	Klebsiella oxytoca	Hillah public health Lab

The Preparation of the Bacterial Inoculums

The nutrient broth media was used for the purpose of preparation of the inoculums in test tubes of 10 ml per tube and sterilize in autoclave at 121°C for 15 min. Inoculums was prepared for each bacterial isolates diluted the cell suspension with sterile normal saline to obtain final concentration by comparison with 0.5 McFarland.

Antibacterial Activity of ZnO Nan particles Against Pathogenic Bacteria

Inhibitory effect has been tested for ZnO NPs. against bacterial isolates shown in table (1), with a concentrations (100, 200, 400, 600, 800, and 1000) µg/ ml. using the:

Determination of the Minimum Inhibitory Concentrations (MIC)

The minimum inhibitory concentrations (MIC) were determined by the micro well dilution method with some modifications. This test was performed in sterile flat bottom 96 well micro test plates. 150 µl volume of Mueller Hinton broth was dispensed into each well and 20 µl of various concentrations

of the nanoparticle was added along with 30µl of the test organism suspension. The final volume in each well was 200 µl (150 µl Mueller Hinton broth, 30 µl of the test organism suspension, and 20 µl nano particle).Therese control wells were maintained for each test batch the positive control (media and test organism), the negative control

(media and nanoparticles) and third control was the media only Plates were then incubated at 37°C for 24 h. The MIC was determined using the spectrophotometer at wavelength 600 nm after incubation [12]

Determination of the Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) was determined from all wells showing no growth as well as from the lowest concentration showing growth in the MIC assay for all the samples. Bacterial cells from the MIC test plate were sub cultured on freshly prepared solid nutrient agar plates by making streaks on the surface of the agar. The plates were incubated at 37°C for 24 h overnight. Plates that did not show growth was considered to be the MBC for the nanoparticle used [13].

Results and Discussion

The white precipitate at the bottom of the flask indicates all ZnO NPs are deposited Fig.1

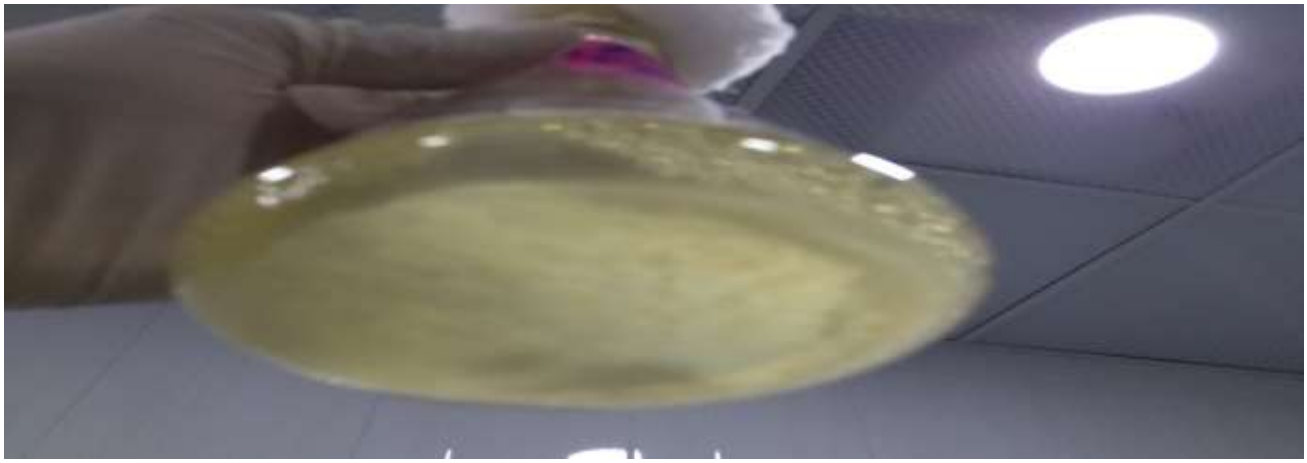

Fig.1: Precipitated of biologically ZnO NPs at the bottom of flask

Fig.2 represents XRD patterns of the bio synthesis ZnO NPs synthesized using *bacillus subtilis*. XRD investigation reflects that synthesized nanoparticles were pure and crystalline in nature.

The detected diffraction peaks of ZnO at 2-theta = 31.72° ,34.38° and 36.26° are associated with (100),(002) and (110) all the reflections can be assigned to the stander powder pattern of ZnO [5].

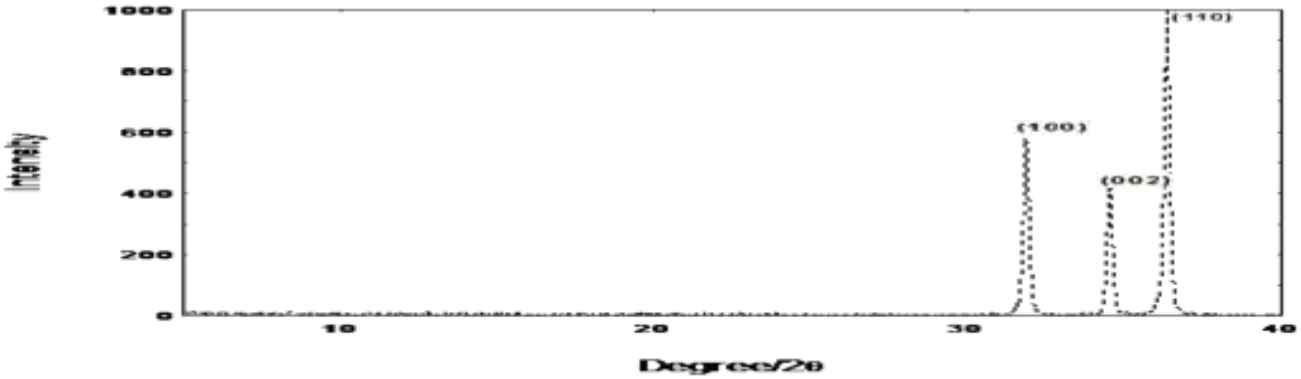

Fig 2: XRD pattern of biologically ZnO nanoparticles using *Bacillus subtilis*

Optical properties of the ZnO NPs were shown by UV–v is spectroscopy as in fig. 3. It is clear that absorption peak at 385 nm [6] shows the existence of ZnO NPs. The field-emission scanning electron microscopy characteristics of ZnO deposited on Ga N and Si substrates were measured in a two-parallel-plate fig. 4. The vacuum chamber was evacuated to a base

pressure of 1.5 x 10^{-5} Torr. The voltage was swept by automatic sweep option of source meter from 0 to 1000 V. TEM image of the synthesized ZnO NPs at 50 nm scales are shown in the Fig.5. It was seen that ZnO NPs are poly dispersed and roughly spherical in shape with maximum particles in size range within 7-19nm.

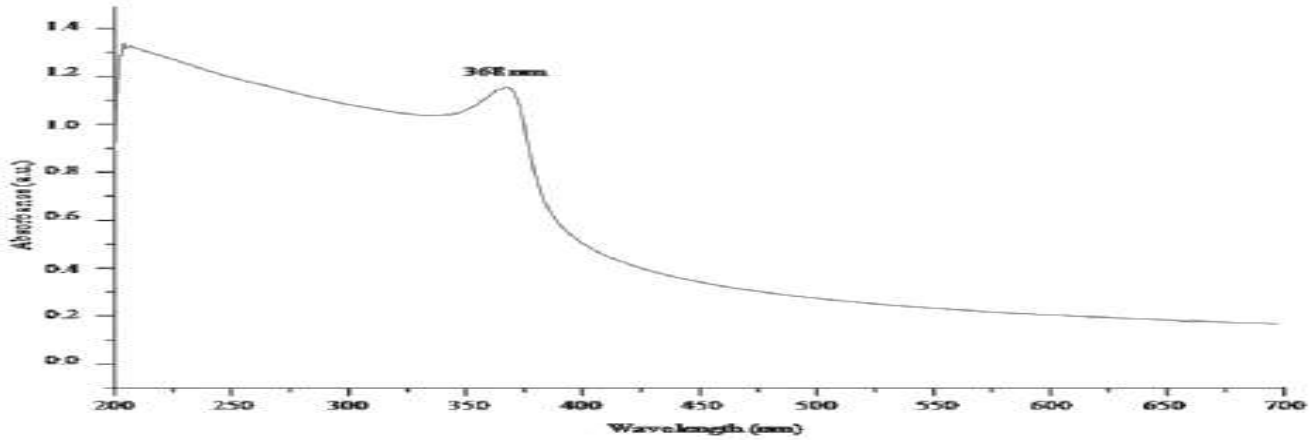

Fig. 3 UV–v is spectroscopy of biologically ZnO nanoparticles using *Bacillus subtilis*

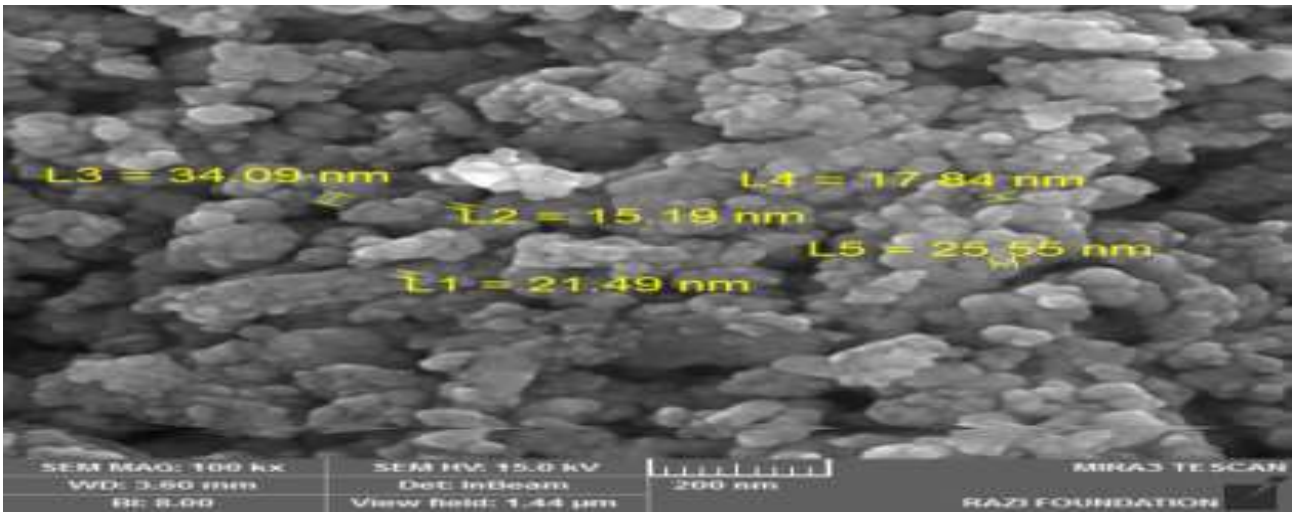

Fig.4 FE-SEM of biologically ZnO nanoparticles using *Bacillus subtilis*

An FTIR spectrum is used to access the details of functional groups involved in the biosynthesis of ZnO NPs. The gotten FTIR spectra of ZnO NPs displayed a clear package located at 400-500 cm^{-1} due to vibration metal association Zn-O [5]as shown in Fig.6.

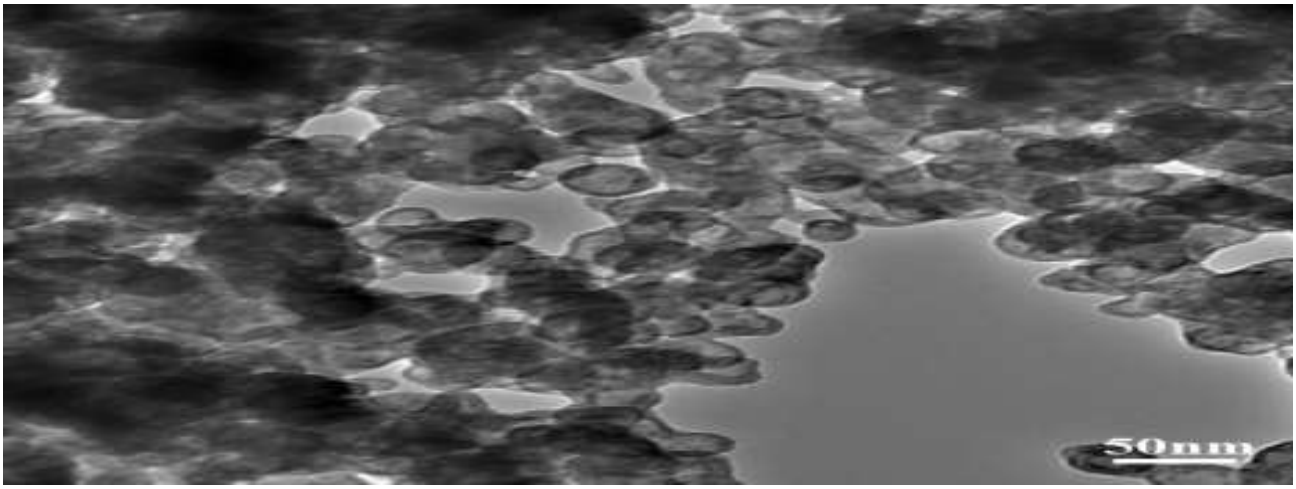

Fig.5 TEM of biologically ZnO nanoparticles using *Bacillus subtilis*

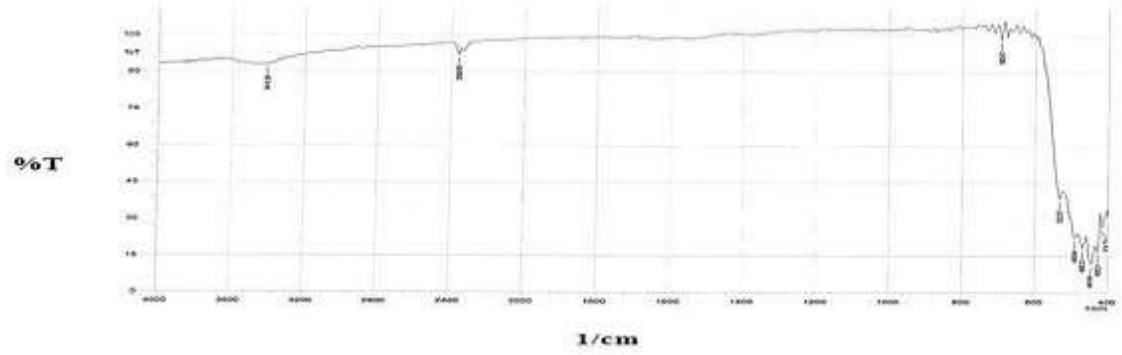

Fig.6 FTIR of biologically ZnO nanoparticles using *Bacillus subtilis*

The Anti-Bacterial Activity of Nan particles against MDR Bacteria

The antibacterial activity of bio synthesis ZnO NP was examined against multidrug Gram-negative bacteria, and one-multi drug Gram-positive bacteria. The first step in this experiment and due to the wide ranges of concentrations used in this experiment.

We forced to choose the MIC and MBC concentration for each nanoparticles by using the broth micro dilution method using the spectrophotometer at wavelength 600 nm after incubation, the calculation was done as show in Figure (7).

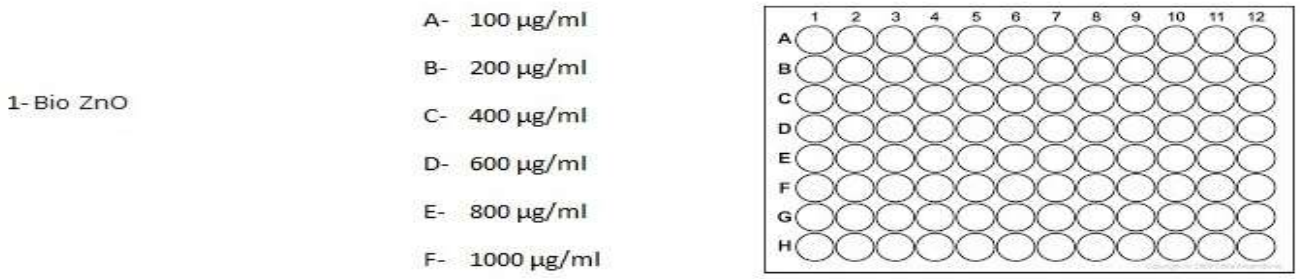

Fig 7: Micro titer plate

After this the nanoparticles minimum inhibition concentration (MIC) was made by broth micro dilution method. 0.5 McFarland's Standard suspension of bacterial was pipetted into a micro titer plate. The bacterial isolates were incubated for 24 hours at 37C° with thoursee-fold replications and for Minimum bactericidal concentration (MBC) Samples from wells used in broth micro dilution assay that did not show any observable growing after the 24 hours incubation was sub cultured onto nutrient agar plates.

The MBC was documented as the lowest concentration of the nanoparticles that did not allow a single colony to grow on the agar after further 24 hours incubation.

Table 2: The minimum inhibitory concentrations and the minimum bactericidal concentration of synthesized nanoparticles

Bacterial isolation	ZnO NP.	
	MIC µg/ml	**MBC** µg/ml
Klebsiella oxytoca	100	200
Methicillin-resistant *staphylococcus aureus* (MRSA)	600	800
Pseudomonas aeruginosa	200	400
Escherichia coli	600	600

It's clear from the table (2) the biologically ZnO nanoparticle was showed various MIC concentration among bacterial strains, the 600 µg/ml was showed inhibition activity against Methicillin-resistant *staphylococcus aureus* (MRSA)and *Escherichia coli* while the 100 µg/ml was worked on *Klebsiella oxytoca* and 200 µg/ml on *Pseudomonas aeruginosa.*

While the MBC concentration of biologically ZnO nanoparticle was showed various concentrations against MDR bacterial strains under the study, the 800 µg/ml was showed inhibition activity against Methicillin-resistant *staphylococcus aureus* (MRSA) and 600 µg/ml against *Escherichia coli* while the 200 µg/ml was worked on *Klebsiella oxytoca* and 400 µg/ml on *Pseudomonas aeruginosa.* [14]

The ZnO nanoparticles were found to show potential antibacterial activity against tested bacterial stain *Klebsiella oxytoca.* So the results completely agreed with study mentioned above.

The ZnO NPs was exhibited antibacterial activity against MRSA [7] so, the result obtained agree with this findings. The proposed two imaginable mechanisms for the antibacterial action of zinc oxide nanoparticles to bacteria are first, creation of augmented levels of ROS mostly hydroxyl radical and singlet oxygen which destruction the bacterial cell wall.

Second nanoparticles deposition on bacterial surface or nanoparticles aggregation either in the periplasm region or in the cytoplasm initiating distraction of cellular function and membranes disorganization.

Also the result showed the same result as in [8]shown that ZnO nanoparticles had antibacterial activity against bacteria with ESBL and Amp-C.

Interestingly, synthesized ZnO non material's [15]exhibits an excellent bactericidal effect against human pathogens, which clearly depicts its suitability to use in diverse no medicinal applications.

According to [9], Antibacterial activity for nanoparticle ZnO against *P. aeruginosa* isolates was measured the zone of inhibition was correlated positively with the concentration so the result agree with above study with one exception the concentration that used in above mentioned study was a little because the bacteria is not MDR pathogenic .

Additionally,[16,17]was showed that the biosynthesis of zinc oxide nanoparticles (ZnO NPs) using culture supernatant of endophytic bacterial isolate *Sphingo bacterium thalpophilum* had showed improved antibacterial activity against *P. aeruginosa.*

Similarly the results completely agree with [10].The result showed that the ZnO has antibacterial activity against *Escherichia coli* bacteria than others.

Also the result came in parallel with result obtain by [11] shown that the Antimicrobial trials established that 20 µg/mL of Au/TiO2 had an recognizable antimicrobial activity, whereas a dose of as low as 10 µg/mL of combined Ag/TiO2 Nano doping displays brilliant antibacterial ability, since the electrical attraction between the highly positive charged Nano doping particles and bacteria with its negative charge supports the communication between silver nanoparticles and bacterial cells.

Conclusion

To accomplish we have used strange, cheap, nontoxic, ecofriendly and richly obtainable *bacillus subtilis* for the quick synthesis of ZnO NPs in the range of 7–19 nm.

The nanoparticles synthesized are temperately stable and this technique has benefit over the other procedures, as the bacteria used here is nonpathogenic and Generally Recognized asSafe (GRAS).

So, our results could be targeted for utilization of *bacillus subtilis* in the biosynthesis of nanoparticles.

References

1. Selvarajan Ea MV (2013) Biosynthesis and characterization of ZnO nanoparticles using Lactobacillus plantarum VITES07. Materials Letters, **112**: 180-182.
2. Selvarajan E, V Mohanasrinivasan (2013) Biosynthesis and characterization of ZnO nanoparticles using Lactobacillus plantarum VITES07. Materials Letters, **112**: 180-182.
3. Jayaseelan CA, A VishnuKirthia, A Marimuthua, S Santhoshkumara, T Bagavana, A Gauravb, K Karthikb, L BhaskaraRao KV (2012) Novel microbial route to synthesize ZnO nanoparticles using Aeromonas hydrophila and their activity against pathogenic bacteria and fungi. Spectrochimica Acta Part **90**: 78-84.

4. Mudit MJ, SP Suneet, KS Selvarajan,E Subathoursadevi, C Mohanasrinivasan V (2013) Studies on the Inhibitory Activity of Biologically Synthesized and Characterized Zinc Oxide Nanoparticles using Lactobacillus sporogens against Staphylococcus aureus. Journal of Pure And Applied Microbiology **7(2)**.

5. Bashi AMH, MZ Zainal, Z Tichit D (2013) Synthesis and controlled release properties of 2,4-dichlorophenoxy acetate–zinc layered hydroxide nanohybrid. Journal of Solid State Chemistry, 203: 19–24.

6. Vanheusden KW, WL Seager,CH Tallant, DR Voigt, JA Gande BE (1996) Mechanisms behind green photoluminescence in ZnO phosphor powders. Journal of AppliedPhysics, 79: 7983–90.

7. Seyedeh AA et al (2016) Antibacterial activity of Zinc oxide nanoparticles on Staphylococcus aureus. International Journal of Advanced Biotechnology and Research 2016. **7(3)**: p. 569-1575.

8. Asfia S et al (2015) Antibacterial Activity of ZnO Nanoparticles against ESBL and Amp-C Producing Gram Negative Isolates from Superficial Wound Infections. International Journal of Current Microbiology and Applied Sciences, 38-47.

9. Noor HA, DS Halah (2016) Antibacterial activity of modified zinc oxide nanoparticles against Pseudomonas aeruginosa isolates of burn infections. j. World Scientific News **33**: 1-14.

10. Al-Hartomy OA, M Mujahid (2014) Synthesis and characterization of zinc oxide nanosheets for protection against multiple drug resistance bacteria (MDR). American Journal of Biological Chemistry, 2(3): 17-22.

11. Shao FC et al (2010) Large Scale Photochemical Synthesis of M@TiO2 Nanocomposites (M = Ag, Pd, Au, Pt) and Their Optical Properties, CO Oxidation Performance, and Antibacterial Effect. J.nano res. **3**: 244–255.

12. Frey FM, Meyers R (2010) Antibacterial Activity of Traditional Medicinal Plants Used By Haudenosaunee Peoples of New York State. Bmc Complementary and Alternative Medicine, 10, 64-73.

13. 1Akinyemi KO, Oladapo O, Okwara CE, Ibe C, Fasure KA (2005) Screening Of Crude Extracts Of Six Medicinal Plants Used In South-West Nigerian Unorthodox Medicine For Anti-Methicillin Resistant Staphylococcus Aureus Activity. Bmc Complementary and Alternative Medicine, 5, 6-12.

14. Padmavathy N, Vijayaraghavan R (2008) Enhanced Bioactivity Of Zno Nanoparticles—An Antimicrobial Study. Sci. Technol. Adv. Mater. , 9

15. Sathishkumar G, Rajkuberan C, Manikandan K, Prabukumar S, DanielJohn J, Sivaramakrishnan S (2017) Facile biosynthesis of antimicrobial zinc oxide (ZnO) nanoflakes using leaf extract of Couroupita guianensis Aubl. Materials Letters. 188: 383-386.

16. Rajabairavi N, Raju CS Karthikeyan, C Varutharaju, K Nethaji, S Hameed, ASH Shajahan A (2017) Biosynthesis of novel zinc oxide nanoparticles (ZnO NPs) using endophytic bacteria Sphingobacterium thalpophilum. International Conference on Recent Trends in Materials Science and Applications. 189: 245-254.

17. Alkaim, AF, Alrobayi EM, Algubili AM, Aljeboree AM, (2017) Synthesis, characterization, and photocatalytic activity of sonochemical/hydration–dehydration prepared ZnO rod-like architecture nano/microstructures assisted by a biotemplate. Environmental Technology (United Kingdom), 38: 2119-2129.

