Antibacterial Efficacy of Biosynthetic Zinc Oxide Nanoparticles by Lactobacillus Plantarum Combined with Poly-β-hydroxybutyrate Against Pathogenic Bacteria

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Abstract

The good capability of zinc-tolerant probiotic of *Lactobacillus plantarum* tolerating high concentrations of $Zinc^{+2}$ and producing Zinc Oxide Nanoparticles highlights the unique characteristics of these bacteria as a natural microbial cell nano-factory for a more efficient and environmentally friendly process of biosynthesis of these nanoparticles. The morphological and structural properties of Zinc Oxide Nanoparticles were determined by X-ray diffraction, Atomic Force Microscope and Field Emission Scanning Electron Microscope showed that the synthesized nanoparticles were crystalline, moderately stable, roughly spherical, hexonal in shape and pure

We examined the antibacterial activity of the Zinc Oxide Nanoparticles and Zinc Oxide Nanoparticles/ Poly-β- hydroxybutyrate bionanocomposite on some pathogenic bacteria .It is clear in this the study that Zinc Oxide Nanoparticles/Poly-β-hydroxybutyrate bionanocomposite has stronger inhibitory effect on pathogenic bacteria compared with Zinc Oxide Nanoparticles only because the bionanocomposites exhibited reduced water uptake and superior gas as well as vapour barrier properties and causes increasing damage of the pathogenic bacteria.

Keywords: *ZnONPs, Lactobacillus plantarum, PHB, antibacterial efficacy and bionanocomposite.*

Introduction

Zinc oxide nanoparticles have gained worldwide interest as multifunctional nanoparticles because of their distinctive features of being versatile semi- conductors and piezoelectric properties[1]. Microbial metal nanoparticles synthesis has recently been widely used due to their low cost, biocompatibility and eco-friendliness[2]. ZnO has many important features like chemical and physical stability, high catalysis and efficient antibacterial activity[3]. Symbiotic microorganisms may use NPs as safe source. Microbial nanoparticles synthesis has more benefits than other chemical and physical method. Nanoparticles have many applications in medicine .In addition, the bacterial nanoparticles may also be used for controlling human bacterial pathogens[4]. Among the micro-organisms, lactic acid bacteria (LAB) receive substantial attention because of their safe handling and food-grade status, which are "generally recognized as safe" (GRAS) in the production and preservation of food[5]. A low-cost, unreported and easy method for the biosynthesis of ZnONPs by using reproducible bacteria, *Lactobacillus plantarum* as an eco-friendly reduction and capping agent is described in our current study. *Lactobacillus plantarum* is a non- pathogenic, gram-positive, facultative anaerobic bacteria and is the largest of all lactic acid bacteria in its genome. *Lactobacillus plantarum* has a negative electrokinetic potential; which attracts cations easily and this step works as a trigger for the ZnONPs biosynthesis[6]. Poly-β-hydroxybutyrate is a biodegradable thermoplastic polyester that can be used in medicine, agriculture, etc.. Because of its non-toxic, biodegradable and biocompatible nature, poly-3-hydroxybutyrate (PHB) is considered as an ideal drug carriers[7]. Using solution casting technique, bionanocomposites based on PHB incorporating different content of ZnONPs were prepared. The nanoparticles are distributed without the need for surfactants or binding agents within the biopolymer[8].

Methodology

Bacterial Isolate: *Lactobacillus Plantarum* was selected as a biological model for the synthesis of ZnONPs because it is more efficient for biosynthesis of ZnONPs .We have obtained this isolate from the dairy products which is stored in the advanced Microbiology Lab./University of Babylon. We did culture to the isolate for 24hr. on MRS agar at 38°C. as well as it is diagnosed by Vitek2 system.

Zinc Oxide Nanoparticles' Biosynthesis by *Lactobacillus plantarum:* The pure culture of *Lactobacillus plantarum* was inoculated in the flask containing MRS broth that sterilized by autoclave and incubated at 37° C for 24hr. at 100rpm. After the incubation period, we did Centrifuge 5000 rpm. for 25 min. then we took the supernatant .The pH of the supernatant was regulated by 0.4 M NaOH to delay the transformation process (the pH of the supernatant is acidic 4.7 to be neutral we added the NaOH to reach pH 7 to eliminate the influence of organic acids). 28.8 g. 0.1 M $ZnSO_4.7H_2O$ dissolved in 1000 ml of distilled water, was added to 250 ml of the supernatant and then heated by a water bath of up to 85 ° C for 5-10 min. A white precipitate appears at the bottom of the flask indicates the process of transformation .Then the flask was Incubate at 37 ° C for 12 hr., all the particles are accumulated at the bottom of the flask. In order to separate the white precipitate, the product was Centrifuged at 6000 rpm for 20 min. and washed with D.W. then the process was repeated 3 times to get pure products followed by drying at 60 ° C in a hot air oven for 4 hr[9].

The instruments that used in determining the properties of ZnO nanoparticles biosynthetic by *lactobacillus plantarum* :-

- X-ray diffraction analysis (XRD).
- Atomic Force Microscope (AFM).
- Field Emission - Scanning Electron Microscopy (FESEM).

Preparation of Zinc Oxide Nanoparticles

Reinforced Poly-β-hydroxybutyrate Films: 0.3gm. Poly-β hydroxybutyrate (manufactured by SIGMA-ALDRICH company/Germany) was put into the ZnONPs mixed solution 0.18 gram of ZnONPs after that putting them into scuttle bottle containing 30 ml. chloroform then stirring by ultrasonic bath device for 30 min. continuing the process until achieving the homogeneous solution and verified the homogeneity by physical observation. Then the films were poured into glass petri dish at room temperature 30 °C and left for 48 hrs. for evaporation of chloroform [10]. In this study we use different weights of PHB (0.1, 0.2, 0.3 and 0.03gm.) while using 0.18 and 0.3 gm. of ZnONPs in the preparation of ZnONPs reinforced P(βHB) Films.

Antibacterial efficacy of ZnONP and PHB-ZnONPs bionanocomposite.

Disc Diffusion Method.

This method was accomplish on Muller Hinton media as follows:

1. Concentrations were taken from each bacterial isolates and compared to McFarland solution to get the right concentration for each of them.
2. The appropriate concentration 0.1 ml of each bacterial isolates were added to dishes containing Muller Hinton agar is spread on the surface of the dish-by spreader and left the dishes for an hour.
3. PHB-ZnONPs bionanocomposites were made (3.2.11) in cork borers each 6 mm diameter . it was equal distance between the film and well. Table 1 shows the concentrations of ZnONPs and ZnONPs/PHB used in the present study.

Table (1): Different concentrations for ZnONPs and ZnONPs/PHB

	ZnONPs/ PHB wt.%	ZnONPs + PHB Concentration (mg/m)		ZnONPs Concentration (mg/ml)
1	0.18/0.1= 1.8	0.20	1	0.6
2	0.18/0.2= 0.9	0.10		
3	0.18/0.3= 0.6	0.07		
4	0.3/0.03= 10	1.25	2	1.04

4. The nanoparticles were dissolved in distal water to get a final concentrations, and dunk ZnO NPs with filter paper .

5. After incubation at 37°C for 24 hrs., the PHB- ZnO nanocomposite film and nanoparticles inhibition zones were measured by a ruler[11].

Results and Discussion

Zinc Oxide Nanoparticles biosynthesis by

Lactobacillus plantarum: The biosynthesis of nanoparticles from *Lactobacillus plantarum* has been confirmed by observing a change in solution color during the synthesis of ZnONPs that explains the reduction of ZnO into ZnONPs during exposure to bacterial extract followed by a change in color from brown to yellow during 24hr.,this result was compatible with the study done by [12].

The deposition of the white color at the bottom of the flask by combining *Lactobacillus plantarum* with $ZnSO_4.7H_2O$, is agreed with the results reported through [13-15]. *Lactobacillus plantarum* has a major role in the production of Zn-ONPs. It is possibly because this bacteria possesses negative electro-kinetic potential which attracts the cations readily and triggers the synthesis of nanoparticles. Furthermore, *Lactobacillus* has the capacity to grow even in the presence of oxygen allowing more capability of metabolic growth. In addition, the presence of glucose in the MRS media used for Zn-ONPs synthesis tends to lower the potential for oxidation- reduction. Energy producing glucose (which regulates the value of rH_2), medium pH ionic status and complete oxidation reduction potential (rH_2) partially controlled by sodium hydroxide, all of these factors cumulatively negotiate ZnONPs synthesis in the presence of *Lactobacillus* [9]. the final stage of ZnONPs biosynthesis by *Lactobacillus plantarum* after the process of drying these nanoparticles in the oven .

Morphological & Structural properties of Zn-ONPs biosynthesized by *Lactobacillus plantarum*: Moderately stable ZnONPs have been synthesized using *Lactobacillus plantarum* .The effect of reaction time plays a vital role in the morphology of nanoparticles .The Field Emission Scanning Electron Microscopy image of the ZnONPs have shown spherical clusters of the nanoparticles as in figure 1 shows the diameters of ZnONPs (21.75 nm, 24.13 nm, 25.77 nm), indicating the diameters of NPs were accurate and appropriate as ZnONPs.

X-Ray diffraction analysis (hexagonal phase) shows that the synthesized nanoparticles were crystalline and pure in nature. The peaks at 2θ = 32.01, 45.28, 56.41, 66.12 and 75.03 were respection lines of spherical Zn-ONPs respectively, figure 1. The average particle size of ZnONP was determined by applying the Scherrer equation, The maximum diameter measured for particles is 5.5 nm., this result was agreed with the results reported through [16,17].

Atomic Force Microscope analysis of synthesized ZnONPs was carried out to assess their morphology and size range. The 2-D and 3-D images of AFM, figure 3, showed that most of the nanoparticles are spherical in shape and some of the agglomerations were present in the background of the nanoparticles. AFM analyses revealed that obtained nanoparticles were in a hexagonal, polydispersed, nearly spherical in shape, these results were compatible with the study of [18].

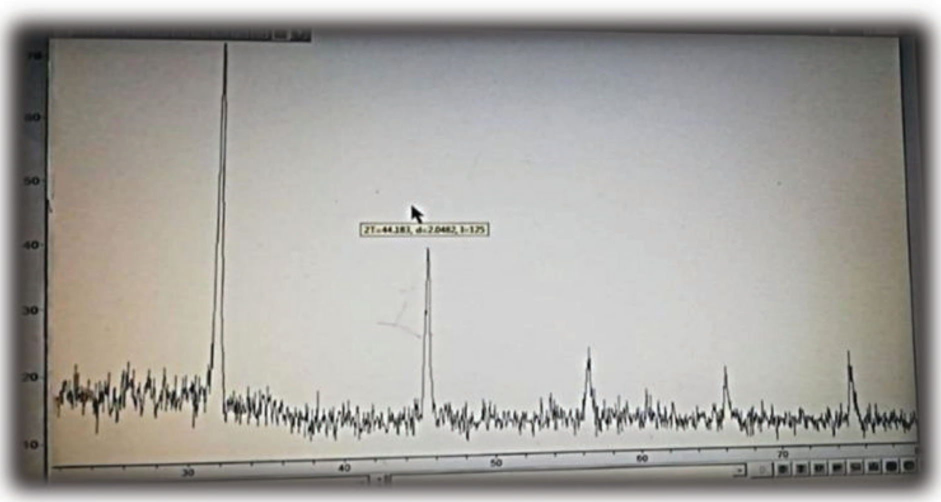

Figure 1. X-Ray diffraction (XRD) Microscopy (FESEM)

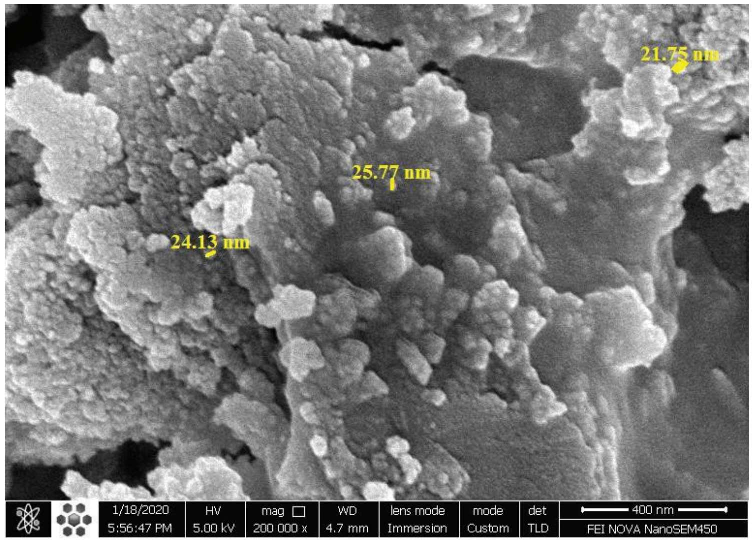

Figure 2. Field Emission Scanning Electron

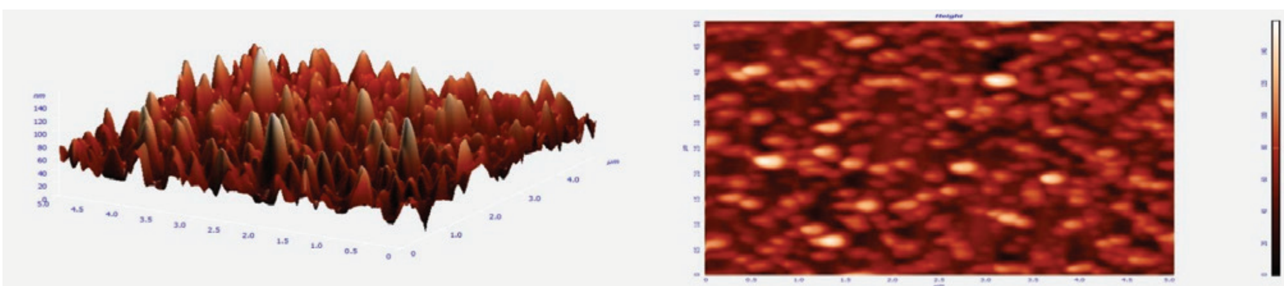

Figure 3. 2D and 3D images of ZnONPs biosynthesized by *Lactobacillus plantarum*. by Atomic Force Microscope (AFM).

Figure (1) shown properties of ZnO nanoparticles biosynthesized by *Lactobacillus plantarum*.

Anti-Bacterial Efficacy of Zn-ONPs and PHB/Zn-ONPs against Pathogenic Bacteria.

The antibacterial efficacy of biosynthesis ZnONPs and PHB/ZnONPs bionanocomposites were examined against four pathogenic bacteria as shown in table 2**.**

Table (2): Pathogenic bacterial isolates obtained from Babylon hospitals.

Bacterial isolates	**Gram stain**	**Type of sample**	**Source Obtained**
Escherichia coli.	Negative	Urine	Merjan Medical City
Klebsiella Pneumoniae.	Negative	Swab (sputum)	Al-Hilla teaching hospital
Staphylococcus epidermidis	Positive	Blood	Merjan Medical City
Staphylococcus aureus	Positive	Swab(sputum)	Public health laboratory

The antimicrobial efficacy was due to cell membrane damage, leading to cell contents leakage and cell death. While the exact mechanism of action is still unclear, the generation of H_2O_2 (a potent oxidizing agent harmful to the cells of living organisms) from the surface of Zinc Oxide has been considered as the main factor of Zn-O reinforced nanocomposites 'antibacterial efficacy [19]. Zn NPs or ZnONPs of extremely low concentrations can't cause toxicity in human systems [20]. The antibacterial efficacy of Zn-ONPs and bionanocomposites Zn-ONPs/PHB were tested using the method of disc diffusion agar, Tables 3 and 4. The existence of an inhibition zone clearly indicated that ZnONPs had an antibacterial effect. As indicated in the study of [21] it was also observed in this study that the growth inhibition was also increased by increasing the concentration of ZnONPs or Zn-ONPs/PHB in the disc. Depending on the type of pathogenic bacteria and concentrations of ZnONPs or ZnONPs/PHB bionanocomposites the inhibition zone was different. In this study, four concentrations of ZnONPs and Zn-ONPs/PHB bionanocomposites were used as in tables 3 and 4 indicate that increasing concentrations of ZnONPs and ZnONPs/PHB bionanocomposites leads to increase growth inhibition of the pathogenic bacteria, but ZnONPs/PHB bionanocomposites has more activity in the inhibitory effect for these pathogenic bacteria,this analysis agreement with previous study [22].

Table (3): Shows the diameter of inhibition zone of ZnONPs for some pathogenic bacteria.

	ZnONPs wt. (g.)	ZnONPs Con. (mg/ml)	ZnONPs(mm) Staph. aureus	ZnONPs (mm) Staph. epidermidis	ZnONPs (mm) K. pneumoniae	ZnONPs(mm) E. coli
1	0.18	0.6	8	24	9	10
2	0.3	1.04	13	29	14	15

Table (4): Shows the diameter of inhibition zone of ZnONPs +PHB bionanocomposites for some pathogenic bacteria.

	ZnONPs/ PHB wt.%	ZnONPs+PHB Con.(mg/ml)	ZnONPs+PHB(mm) Staph. aureus	ZnONPs+PHB(mm) Staph. epidermidis	ZnONPs+PHB(mm) K. pneumoniae	ZnONPs+PHB (mm) E.coli
1	0.18/0.1= 1.8	0.20	12	31	13	16
2	0.18/0.2= 0.9	0.10	10	29	11	14
3	0.18/0.3= 0.6	0.07	9	28	10	13
4	0.3/0.03= 10	1.25	15	34	19	18

Also figure 2 explains the difference in the efficiency of the types of inhibitors for pathogenic bacteria is indicating that the efficiency of ZnONPs/PHB has stronger inhibition because the bionanocomposites exhibited reduced water uptake and superior gas as well as vapour barrier properties compared to ZnONPs and causes increasing damage to *Staph. aureus* .

Conclusion

Our current study for ZnONPS biosynthetic by *Lactobacillus Plantarum* and ZnONPs/PHB bionanocomposite, and using these them as antibiotics give us wonderful results, low complications, very low cost and low resistance in comparison with ordinary antibiotics. This means that ZnONPS and ZnONPS/PHB have high antibacterial efficacy but PHB/ZnONPs bionano- composites were more effective than ZnONPs in inhibition the growth of pathogenic bacteria . The results represent a great potential benefit for a wide numbers of medical applications in the battle against antibiotic-resistant bacterial pathogens.

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Conflict of Interest: None to declare.

Ethical Clearance: All experimental protocols were approved under the College of Biotechnology and all experiments were carried out in accordance with approved guidelines.

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