RESEARCH ARTICLE

Impact of some Environmental Factors on the Activity of Zinc Oxide Nanoparticles fabricated by *Bacillus subtilis*

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ABSTRACT

Nanobiosynthesis has the upper hand over the nonbiological (physical and biological) methods, it is economic, and ecofriendly and sustainable. This study aimed to biosynthesize Zinc oxide nanoparticles by using *Bacillus spp* and evaluate their activity exposure to some environmental factors. The Zinc oxide nanoparticles was evidenced by trans-coloration of the reaction medium from yellowish into turbid yellow. Biogenic nanoparticles have been characterized using Atomic force microscope (AFM), X-ray Diffraction (XRD), Scanning electron microscope (SEM), and Energy dispersive spectroscopy (EDS). It is found that Zinc-oxide nanoparticles was homogenous nanorods, with size distribution (20–80nm). The average size of biogenic nanoparticles was 26 nm.

Keywords: *Bacillus subtilis*, Nanobiotechnology, SEM, Zinc oxide.

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INTRODUCTION

Nanotechnology is a multi-disciplinary area of science that covers many areas of scientific techniques, like biomedical, pharmaceutical, environmental, and technology, etc.[1] Nanotechnology is become applied now in the medical industries, including pharmaceutical, medical engineering, and tissue engineering; engineering industries including electronics, robotics, and nano-engines. The usage of nanomaterial in the enhancement of delivery systems for various molecules, like proteins, nucleic acids, and plasmids.[2] Nanoparticles has been considered widely throughout the last years. It has been used to deliver drugs to a target tissues and to increase stability against enzymatic degradation.[3] Nanoparticles' unique morpho-dependent properties makes it vital and superior in many zones of human activities. In addition to its green and eco-friendly nanobiosynthesis approaches; biogenic nanoparticles are eco-friendly, biocompatible, and applicable in therapeutic and biomedical applications.[1] Nowadays, the evolvement of biotic systems especially the microorganisms, has employed as an innovative pattern for the production of nanomaterials; biological approaches for the synthesis of NPs have the upper hand over chemical and physical methods for their ecofriendly, sustainable, and cost effective. Therefore; the use of eukaryotic and prokaryotic organisms and their extracts in the synthesis of NPs harvest the attention of the researchers in the field of Nanobiotechnology.[4]

Microorganisms isolated from different environments for nanobiosynthesis differ in their cultural and production requirements such as temperature, time, pH, substrate concentration, inoculums size and nutritional requirements. These differences play a determinant role in the characteristics of the biogenic NP yeild. For this reason, each of the mentioned environmental factors must be optimized for each individual microorganism used for the production of particular nanoparticles type, these particles have an interact ability with various biomolecules in various paterns due to their superparamagnetic characteristics, wide choice of surface functionalization and high specific area.[5] The present study is designed to detect ZnO-NPs producer *Bacillus subtilis* isolated from soil, and characterization of biological and environmental properties.

METHODOLOGY

Preparation of Cell Free Supernatant of *B. subtilis*

Bacteria was inoculated separately on a brain heart infusion broth, cultivated at 37°C for 24 hours by two activation**.** After 24 hours of incubation, each bacterial culture was spun at 6000 rpm for 5 minutes at 4ºC to formulate a cell free reaction medium. Pellets of bacteria were discarded and cell free medium were transferred to the reaction flask prepared for nanobiosynthesis process.[6]

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Biosynthesis of ZnO-NPs

Supernatant of bacteria were combined with 1 mM Zn $(CH_3CO_2)_2$ solution at a ratio of 1:1. The suspension was incubated for 48h at 35°C ,200 rpm in shaking incubator. The production of nanoparticles was represented by transcoloration of the media. Then the suspension was spun at 5000 rpm for 15 minutes. The pellet was washed three times, desiccated in a hot air oven at 50°C and sampled for UV, XRD, AFM SEM, and EDS characterization. The excess of NPs yield was stored for further use.[7]

Characterization of Nanoparticles

SEM and EDS Analysis

Shape and size of the biogenic nanoparticles were characterized (SEM) analysis in the main center for tests and consultation, Amirkabir University of Technology, Iran. The microscope was operated in a low vacuum mode, at accelerated voltage 12.5-15 KV with a different amplification powers, spot size 5 and working distances 5–10mm. The composition of specimen was analyzed with EDS in order to indicating and representing analysis with proper condition.[8]

AFM Analysis

Atomic Force Microscopy (AFM) analysis of the biogenic nanoparticles was performed in the Department of Chemistry, College of Science, University of Baghdad.

XRD Analysis

The XRD Analysis of the biogenic nanoparticle have been performed at the main center for tests and consultations, Amirkabir University of Technology, Iran.

Uv-Vis Spectrophotometry

Analysis of Uv-Vis spectrophotometry of the biogenic ZnO-NPs, was performed in the laboratory of solid state at the Department of Physics, Faculty of Science, University of Kufa.

Optimization Conditions for Synthesis of ZnNPs

Temperature: Optimum temperature of the synthesis reaction was determined by constructing a gradient synthesis temperature with precision of 5C° (25, 30, 35, 40, and 45°C) was applied. The other criteria were set at PH 9, and rotation speed 200 rpm. After 48 hours of incubation, the absorbance was recorded at 258 nm.[9]

pH Effect: in order to determine the optimum PH for the nanoparticle synthesis reaction, a gradient PH with precision of 2 pH values (1, 3, 5, 7, 9, 11 and 13) was applied. For these conditions, synthesis reaction was performed at 35°C, and rotation speed 200 rpm. After 48 hours of incubation, the absorbance was read at 258 nm.[9,10]

Optimization of Incubation Time

In order to verify the optimum period for the nanoparticle biosynthesis reaction; the reaction mix was incubated at a gradient time of incubation with precision of 24 hours (24, 48, 72, 96, and 120 hours). At these times, reaction conditions were set at 35°C, and pH 9, and rotation speed 200 rpm. At each time precision, the absorbance was read at 258 nm.[10]

RESULTS

Biosynthesis of Zinc Oxide Nanoparticles

B. subtilis, showed an ability for extracellular ZnONPs biosynthesis using cell free reaction medium after adding Zn $(CH3CO2)_2$ in a concentration 1 mM to the cell free reaction medium, transcoloration of reaction mix from yellowish to turbid yellow indicating the presence of ZnO after incubation at 35°C for 48 hours at pH 9 [Figure 1(a, b)].

Characterization of the Synthesized Nanoparticles

Scanning Electron Microscopy Analysis

The results of SEM showed that green method (biosynthesized using *B. subtilis*) is producing hexagonal nanorods ZnO nanoparticle of varying sizes and homogenous with diameter of (20–80 nm) (Figure 2).

Atomic Force Microscopy

The AFM analysis of biogenic nanoparticles biosynthesized *B. subtilis* revealed nanoparticles' average diameter (65.17 nm) and rough surface. Therefore, etching time and current density can be adjusted to control the size and shape of the structural finish (Figure 3).

X-ray Diffraction

X-ray diffraction image in Figure 4 appears the biosynthesized of zinc oxide NPs where the series of distinguishing peaks

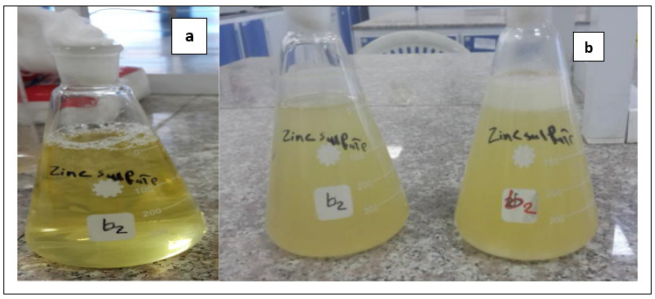

Figure 1: Biosynthesis of ZnO-nanoparticle - supernatant a. before incubation and b. after incubation

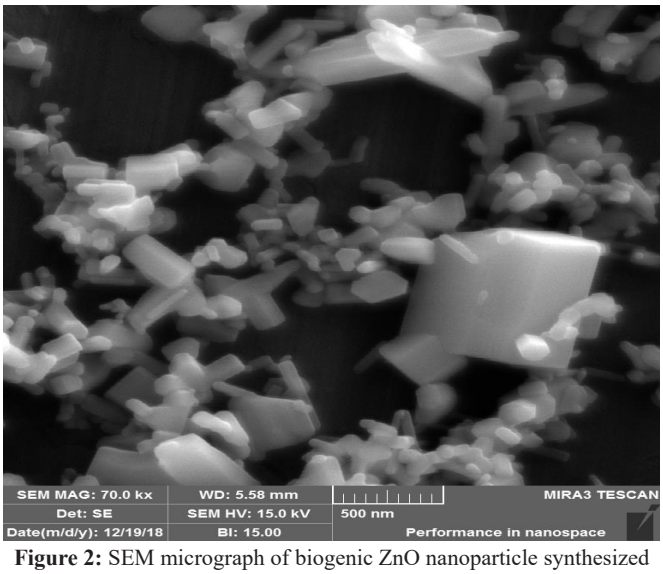

Figure 2: SEM micrograph of biogenic ZnO nanoparticle synthesized

at 2θ and Bragg reflection as mentioned in Table 1, which comparable with pattern references magnitude of XRD infers that the biogenic are cubic back in creation. Sherrer's equation calculations revealed ZnONPs average particle size about 18.17 nm.

UV spectroscopy

The biosynthesized zinc oxide nanoparticles band was indicated by using the UV-Visible scanning (Figure 5) at 258 nm. That refers to dispersion of particles in the aqueous solution.

Figure 6 shows the FT-IR spectrum of ZnO NPs synthesized by bacteria that determined by the band between 800–400 cm^{-1} for zinc oxide, in addition, the specific vibrations for the Zn-O bonds are allocated at 623 and 582 cm^{-1} where these bands are disappearing in spectrum of zinc oxide also the band at 1585 for Zn stretching vibration is missing in zinc oxide spectrum.

Optimization Conditions for Synthesis of ZnONPs

Effect of Temperature: As it is biological approach, nanobiosynthesis is highly affected by the reaction temperature, and by extension the quantity and quality of the ZnNPs. The absorbance spectra obtained by increasing the temperature reaction from 25°C to 45°C Figure 7, indicated an optimal temperature at 35°C with pH 9 and incubation time 48 hours.

Effect of pH

As it is biological approach, nanobiosynthesis is highly affected by the pH of the reaction mix. By applying a gradient pH values ranging from 1–13, Absorbance spectra of the UV-spectroscopy revealed maximum absorbance at pH 9, at temperature 35°C and incubation time 48 hours (Figure 8).

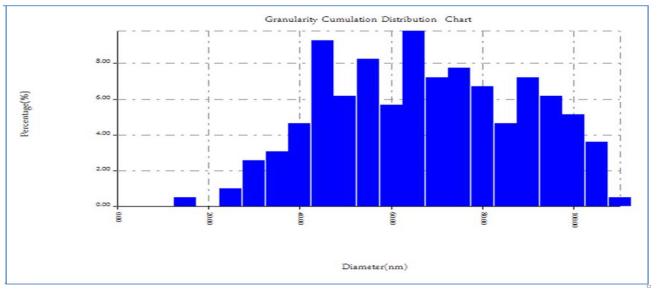

Figure 3: AFM analysis of biosynthesized ZnO nanoparticle

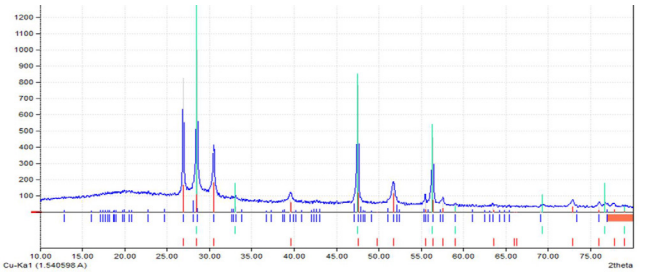

Figure 4: XRD analysis the size of Biosynthesized ZnO nanoparticles

Table 1: Summary of the average particle size projected from XRD outlines by means of the Scherer formula of biosynthesized ZnO NPs.

Sample	*2θ*	*FWHM (deg^0)*	*Particle size (nm)*	*Average particle size*
	26.22	0.614	13.89	
	41.14	0.409	21.69	
	43.26	0.614	14.55	
	47.47	0.614	14.77	
ZnO	50.74	0.409	22.47	18.17
	57.56	0.409	23.16	
	61.52	0.358	26.99	
	69.18	0.818	12.33	
	71.81	0.748	13.71	

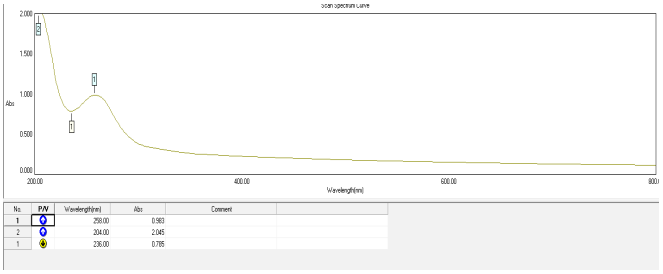

Figure 5: Absorbance spectra of zinc oxide nanoparticles

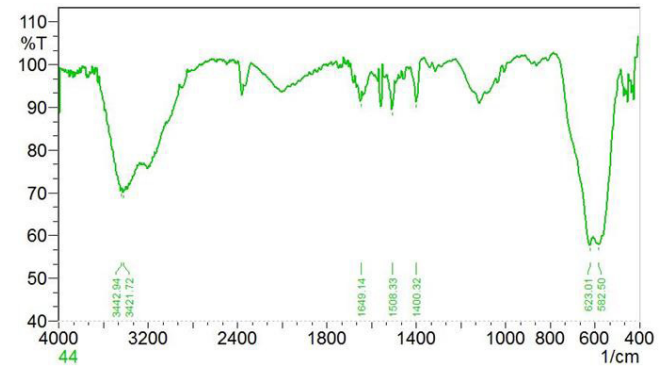

Figure 6: FTIR image of ZnO_4

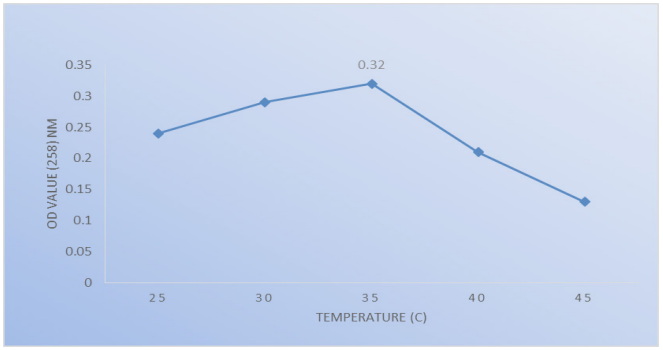

Figure 7: Temperature effect on zinc oxide nanoparticle production

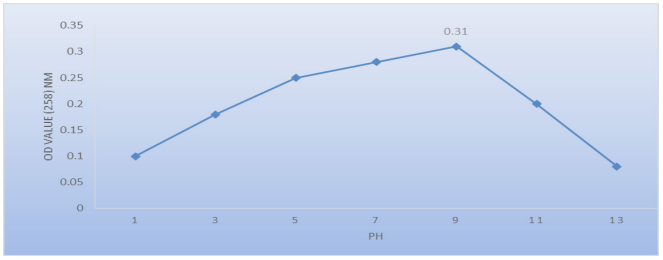

Figure 8 : pH effect on zinc oxide nanoparticle production

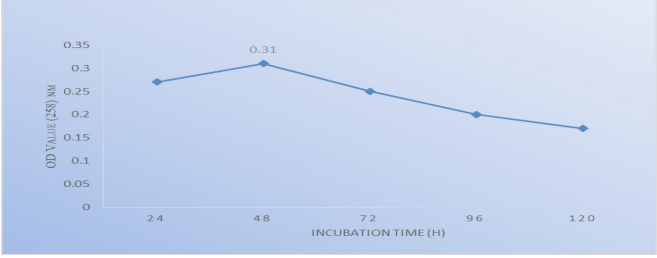

Figure 9: incubation time effect on zinc oxide nanoparticle production

Effect of Incubation Time

By incubation of the reaction mix for a gradient time interval, UV-spectroscopy revealed maximum absorbance at incubation time around 48 hours (Figure 9).

DISCUSSION

Biosynthesis of Zinc Oxide Nanoparticles

This study was performed on the *B. subtilis* obtained from lab. Of microbiology, diagnosed and confirmed on the basis of biochemical characteristics.

Nanoparticles production is regarded as a survival mechanism implemented by many species of soil microorganisms to eliminate competition, and colonize a niche.[11]

This investigation was accomplished to evaluate the biosynthesis and characterization of ZnO NPs produced by soil *B. subtilis* that have potential for production of nanoparticles. Living organisms evolved machineries for detoxification of toxic metals, by isolation in the cytoplasm, exclusion by the cell wall, or transmuting it (e.g., by Red-Ox reactions) into unhazardous construct.[12] Recently, it has been established that microbes have been explored as biological industrial units for synthesis of metallic nanoparticles.[13-15]

Zinc oxide nanoparticle yield was indicated by medium transcoloration from willowish in to turbid yellow as a result of $Zn(CH3CO2)_2$ reduction into ZnO NPs by means of the chemically complex reducing agents (amino acid, polysaccharides, proteins, enzymes, and other biomolecules) present in the cell-free supernatant which were designated as safe, and ecofriendly.[16,17,18]

The characteristic color of the metallic nanoparticles caused by surface plasmon resonance (SPR) as a result of the entire conduction band free electrons' coherent excitation.[19]

Organisms that established "Zinc resistance machinery", are potent for the fabrication of Zinc nanoparticles. Metabolites of the microorganisms present in the cell free supernatant may act as a catalyst and capping mediators for NPs in the reaction medium.[20]

Studies suggested that nicotinamide adenine dinucleotide (NADH-) and NADH-dependent reductases are the crucial mediators in the metallic nanobiosynthesis. Transporting of electrons from NADH (the electron carrier) catalyzed by NADH-dependent reductase thought to be trigger reduction in the reaction mix.[21,22]

Optimization Conditions for Synthesis

The morphology of the biogenic NPs can be modulated by regulating the conditions of the reaction including incubation time, temperature, composition of the culture medium, pH, and the lighting intensity.[23,24] Investigations approved that the characteristics of biogenic ZnO-NPs fabricated by *B. subtilis* could be regulated by adjusting variety of factors, e.g., reaction temperature, $Zn(CH3CO2)_2$ concentration and pH.[25] Some studies determined that at 35–37°C, the optimal yield of biogenic nanoparticles was obtained, and approved that acidic pH decreases the NPs synthesis as a result of less amounts of hydroxyl ion (-OH) that is crucial for the metal ions reduction, and so reduced nucleation for development of Zinc oxide crystals, on which, incoming atoms of Zinc deposit to form larger sized particles. At Alkaline (pH 9 and more), nucleation foci are developed owing to the abundance of hydroxyl ions, followed by increased deposition kinetics of the atoms.[24,26]

Characterization of Zinc Oxide Nanoparticles

Biogenic ZnO-NPs have been characterized by (SEM, EDS, UV, AFM, and XRD) analysis. In this investigation, the initial evidence of the extracellular ZnO NPs biosynthesis were the transcoloration as the effect of surface Plasmon resonance (SPR).

The *B. subtilis* biosynthesized Zinc nanoparticle with (20-80nm) with variable shapes Zinc present in nanorod form, Other studies by Aldujaili *et al* (2015), and Abdulhassan (2016) on the biosynthesize ZnO nanoparticle from *Bacillus sp.* produce nanoparticle with size (30–100 nm).[27,28] Based on UV spectroscopy, plasmon resonance peak was recorded at 426 nm, this result confirmed metal ion reduction and ZnO-NPs formation. Eftekhari *et al*., (2015), suggested that the absorbance peak of the synthesized ZnO NPs was 425nm which was consistent with the current research.[29]

Energy dispersive spectroscopy analysis perceived the existence of elemental zinc which denoted zinc ions reduction to zinc metal in the medium of the reaction of Zinc oxide NPs from *B. subtilis* was 59.1%. The optical absorbance peak, was detected at 3keV which were a distinctive absorbance of metallic ZnO NPs as shown in Figures 5. The result was parallel with the results of found in different previous studies.[8,30]

X-ray diffraction analysis detect the average size of zinc NPs from *B. subtilis was* 26 nm. The other research showed average diameter of zinc NPs 30–100.[28,30]

Atomic force microscopy analysis expressed the three-dimensional shape of zinc nanoparticles and average diameter 65.17 nm, which belongs to Zinc oxide nanoparticles.

Characterization results (size, shape and dispersity of nanoparticles), ZnNPs synthesis by *B. subtilis was* ideal in terms of quality; minimized size and dispersity. This result might ascribed to the variations in the reduction factors in the cell-free reaction mix that might resumed to the quality and abundance of proteins, enzymes and the rest of biomolecules existed in the cell-free culture medium of every microorganism, as well as their potency to interact with $Zn(CH3CO2)_2$.[6,8]

In conclusion: Freshly *Bacillus sp.* was used in bio-fabrication of ZnNPs. The extracellular excretion of ZnNPs with faster rate stabilized by link the molecules onto the

functional forms. The optimal temperature was observed at 35°C with pH 9 and incubation time 48 hours.

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