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Extracellular Green Synthesis and Characterization of Zinc Oxide Nanoparticles using *Bacillus mycoides*

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A B S T R A C T

The development of biological processes for the synthesis of nano-sized materials is of great importance in the field of nanotechnology. The present study deals with correlation between metal tolerance ability of bacterial isolates and its ability to synthesize Zinc oxide (ZnO) nanoparticles. Twenty-three bacterial cultures were isolated from zinc contaminated soil samples around close vicinity of zinc electroplating industries situated in Satpur MIDC of Nashik city, Maharashtra (INDIA). Among all twenty-three isolates, isolate SB3 was found to have maximum metal tolerance of 1000µg/mL which was identified as *Bacillus mycoides* on basis of morphological and biochemical characters and confirmed using VITEK 2 system. The extracellular biosynthesis of ZnO nanoparticles was performed using *Bacillus mycoides* and 1mM Zinc sulphate heptahydrate as a precursor. Recovery of nanoparticles was done using a mixture of CTAB and Hexane in the ratio 2:1 respectively. The synthesized ZnO nanoparticles were further characterized by using UV- Visible double beam spectroscopy, XRD and SEM confirming the crystalline spherical structure of ZnO nanoparticles having Z-average of 55nm. FTIR Spectroscopy studies indicated the presence of protein moiety for stabilization of formed nanoparticles. Thus, biological synthesis is an ecofriendly and cost-effective way to produce small sized and naturally stable ZnO nanoparticles.

***Key words*:** Zinc oxide nanoparticles, Electroplating industry, Metal tolerance ability, Z-average

Earlier in 1959, Richard Feynman initially presented the concept of nanotechnology in a lecture titled "There's plenty of room at the bottom" at the American Institute of Technology. Then after late in 1900's Taniguchi, a professor in Tokyo University of Science, coined the word "Nanotechnology" with an intension to describe the significance of manufacturing materials at nanometer scale. The term nano is derived from a Greek word "nanos" meaning dwarf and indicates particle size measuring to be one-billionth of a meter (10^{-9}) on scale, thus called Nanoparticles [1-2]. Scientists and researchers created nanotechnology domain owing to its rapid and multidisciplinary advancements in contemporary science and technology, and thus over the past few decades, it has been one of the most emerging topics of science and technology [3]. Among many metal oxide nanoparticles, ZnO nanoparticles have been given increased attention. It is an n-type semiconductor with wide band-gap of 3.36 eV and binding energy of 60meV having unique optical, electrical and chemical properties. It is also non-toxic, less-expensive and easily available [4-6]. Thus, utmost focus of the researchers is to figure out its potential applications [7]. It has wide range of applications in areas like piezoelectric sensors, magnetic materials, gas sensing, antimicrobial, photocatalytic decolorization, cosmetics, etc. [8]. Therefore, much work is focused on the synthesis methodologies for ZnO nanoparticles.

Synthesis of nanoparticles can be done in many ways as like sol-gel method, chemical reactions, solid state reactions, co-precipitation, chemical reduction, photochemical reduction, electrochemical reduction, heat evaporation etc. [9]. Usage of toxic chemicals used in the physical and chemical synthesis of nanoparticles, limits its biomedical applications. Thus, reliable, non-toxic and eco-friendly methods for the synthesis of nanoparticles is of vital importance [10]. Green synthesis of nanoparticles using microorganisms was found to be the best method when compared to other methods [9]. Synthesis of nanoparticles using biological methods helps to annihilate harsh processing conditions which are carried out during physical and chemical synthesis, by enabling the synthesis at optimum pH, temperature, pressure, and concurrently at low cost [11]. Different morphologies of ZnO nanoparticles as like nanorods, nanoplates, hexagonal, tetrapods, nanospheres, can be synthesized by adjusting physical parameters like biological source, pH, temperature, mixing ratio, etc. [12-14]. The present study deals with extracellular synthesis of ZnO nanoparticles using the culture supernatant of *Bacillus mycoides* with $ZnSO_4$

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as a precursor. These synthesized nanoparticles were than extracted using CTAB + Hexane. Formed nanoparticles are confirmed by UV- visible double beam spectroscopy, FTIR and characterized using XRD and SEM.

MATERIALS AND METHODS

Collection of soil samples and Isolation of bacteria

Soil samples were collected from the near vicinity of four zinc electroplating industries; Galaxy Metal Finisher, Pardhi Industries, Sai Metal Electroplating and Satyam Metal finisher, located in Satpur MIDC area of Nashik District, Maharashtra. Collection of samples was done 10-15meters away from the working place. The upper layer of the soil was scrapped off to remove foreign particles and litter before sampling. All soil samples were obtained from 5-10 cm layers below soil surface and 10grams of sample was collected in sterile zipper polythene bag using a trowel and stored in refrigerator at 4°C for further use. Isolation of zinc tolerant bacteria was carried out in Nutrient broth supplemented with 150µg/mL of Zn^{2+} (660mg/L) as $ZnSO_4.7H_2O$ [15]. 10g of each soil sample was inoculated separately in 150 mL of Erlenmeyer flasks containing 90 ml of the above-mentioned media and incubated on shaker (100rpm/min.) at room temperature for 48 hrs. Turbidity was observed in all the flasks after 2 days of incubation. Each enriched culture was serially diluted up to 10^{-6} using sterile distilled water and then isolation of bacteria was done using spread plate method by inoculating 0.1ml aliquot from 10^{-6} dilution tubes in Petri plates containing the above solidified medium. Plates were incubated at room temperature until the growth of distinct colonies was seen. Colonies having different morphology were purified and maintained on nutrient agar slant at 4°C in refrigerator.

Screening of isolates for synthesis of ZnO Nanoparticles

From among twenty-three isolates, a potent isolate for zinc oxide nanoparticle synthesis was screened by determining maximum tolerable concentration (MTC) assay. Tubes containing 9mL nutrient broth were supplemented with 1ml of varying concentrations of zinc sulfate to make final concentration of Zn^{2+} ions in the range of 150, 300, 450, 600, 750, 1000, 1150 and 1300 µg/mL. Tubes without Zn^{2+} ions were used as control. The tubes were inoculated with individual isolate (0.5ml inoculum of 0.1 O.D. adjusted by Spectrophotometer at 600nm) and incubated for 24-48 hours at room temperature. The maximum concentration of Zn^{2+} ions in the medium which allowed the growth of isolates was taken as MTC [16]. Bacterial isolate showing highest MTC value was selected for extracellular synthesis of ZnO nanoparticles.

Characterization of isolated organism

The characterization of isolate SB3 was done by its morphological and biochemical characters and identified as per Bergey's manual of Systematic Bacteriology 9th edition and further confirmed by VITEK 2 system version 05.02.

Synthesis of ZnO Nanoparticles and its recovery using CTAB + Hexane

Stock culture of the *Bacillus mycoides* was inoculated in 100mL of Erlenmeyer flask containing 25 mL of nutrient broth, incubated at room temperature for 24 hours and used as seed culture for further experiment. Broth was then centrifuged at 3000rpm for 10 mins. Pellet was washed thrice with saline in order to remove traces of media. Seed culture of 10mL (10^6cfu mL^{-1}) was inoculated in 500mL Erlenmeyer flask containing 190 mL of Minimal salt medium (g/L) K_2HPO_4-0.1; KH_2PO_4-0.1; $MgSO_4$-0.3; NaCl-0.5 and Glucose-1.0. The culture flask was incubated at room temperature for 24 hours on shaker at 100rpm, culture was centrifuged at 12000 rpm for 10 minutes and the supernatant was used for the synthesis of zinc nanoparticles. The culture supernatant was added to the reaction vessel containing 1mM of zinc sulfate in the ratio 1:1 respectively. The reaction vessel was exposed to sunlight for 2 successive days. White precipitate in the reaction vessel was observed confirming the formation of ZnO nanoparticles. This was then sonicated with Ultrasonicator (Bioera) for 5 mins. with pulse off time of 10 seconds to obtain a colloidal solution [17].

For recovery of zinc nanoparticle from zinc colloidal solution we used phase transfer technique using Cetyltrimethyl ammonium bromide (CTAB) + Hexane. CTAB is an anionic detergent, during shaking of the biphasic mixture, the aqueous solution of zinc nanoparticles forms a complex with the CTAB molecule present in organic phase by forming either coordination bond or weak covalent bond. This process provides sufficient hydrophobic charge over the nanoparticles which helps them to disperse in the organic phase. CTAB act as a capping agent for stability of zinc nanoparticles [18]. For extraction of ZnO nanoparticles, a mixture of 50mL of a 2x 10^4 M solution of CTAB and50mL of 70% hexane was added to 100 ml of the ZnO colloidal solution vigorous shaking of the test-tube resulted in spontaneous generation of two immiscible layers, this results in transfer of the zinc colloidal particles into the organic phase and this was observed by white coloration of the organic phase and corresponding loss in color from the aqueous phase. The CTAB capped ZnO nanoparticles were separated by rotary evaporator and the organic phase was obtained as dry powder and purified by repeated washing with 10% ethanol. The process of washing with ethanol removes uncoordinated CTAB molecules from the zinc oxide nanoparticles. The purified CTAB-Zn nanoparticles powder was resuspended in chloroform for further analysis [19].

Characterization of ZnO nanoparticles

Biologically synthesized ZnO nanoparticles were characterized by using UV-visible spectroscopy. 0.1% (w/v) colloidal suspension of ZnO nanoparticles was analyzed over a range of 200-900nm at a resolution of 1nm using double beam UV –Visible spectrophotometer (BioEra, Instrumentation Lab, K.T.H.M College, Nashik).

Fourier Transform Infrared (FTIR) Spectroscopy was also used to determine the variation in chemical structure of ZnO nanoparticles. Dried powder of ZnO nanoparticles was analyzed on FTIR spectrophotometer (SHIMADZU, Instrument Lab, K.T.H.M. College, Nashik) within a range of 400- 4000 cm^{-1} at a resolution of 4cm^{-1}.

Grain size of ZnO nanoparticles was determined using X- ray diffraction (Rigaku-Mini-Flex, Physics department, SPPU) on a X- ray diffractometer which was operated at a voltage of 25kV and using Cu-Kα radiation (λ= 1.54016 A°). Average grain size (Z-average) was determined using x-ray line broadening using Scherrer equation [20].

Powder of ZnO nanoparticles was suspended into 30% ethanol and subjected to sonication for 5 mins. This sample was used for SEM analysis. The topographical image along with histogram of particle size diameter of ZnO nanoparticles was obtained using Scanning Electron Microscopy (SEM). The image was formed with a resolution of 100nm.

RESULTS AND DISCUSSION

Collection of sample and isolation of bacteria

A total of twenty-three zinc tolerant bacterial isolates were isolated using Nutrient agar supplemented with 660mg L^{-1} of $ZnSO_4.7H_2O$. from four soil samples collected from zinc electroplating industries contaminated with zinc. Among twenty-three isolates, seven, nine, five and two isolates were obtained from soil samples collected from Galaxy Metal Finisher, Pardhi Industries, Sai Metal Electroplating and Satyam Metal finisher respectively. Eman *et al*. [15] collected three sediment and two soil samples from heavy metal contaminated sites and isolated seventy-one bacterial strains on Muller- Hinton agar supplemented with250mg L^{-1} concentration of zinc sulphateheptahydrate.

Screening of isolates for synthesis of ZnO nanoparticles

All the twenty-three isolates were screened for Zn metal tolerance and the result was expressed in MTC. Almost nineteen isolates showed significant tolerance with a varying degree of concentrations. The highest MTC was shown by SB3 which was isolated from Pardhi industry exhibited prominent level of metal tolerance of 1000µg/mL as compared to other isolates. Due to its maximum MTC value, SB3 was selected for synthesis of ZnO nanoparticles. Eman *et al*. [15] estimated the MTC for Zn^{2+} in liquid medium by zinc resistance bacteria with the MTC range between 500µg/mL to 4000µg/mL having the highest MTC value of 2000µg/mL, which was found to be high when compared with that of the present study which was 1000µg/mL. The MTC value of present study was low, this might be because, heavy metals are more toxic in liquid than in solid due to more dispersion in culture. Reports also states that, maximum tolerance values have been found with a varying range which depends upon different strains of isolates and the type of media (solid or liquid) used for study [15].

Characterization of isolated organism

The isolate SB3 produced specific rhizoidal-flat colony, they are Gram positive, non-motile, sub-terminal spore forming rod shaped bacteria, arranged in chain and appears like fungal mycelia. The isolate found to be positive for catalase, oxidase, citrate and Voges Proskauer test, and negative for Indole and Methyl red test, had capacity to ferment glucose, fructose and mannitol but negative for lactose fermentation and was identified as *Bacillus mycoides*. The identification of organism was confirmed using VITEK 2 system version 05.02. by performing thirty-four different biochemical tests (Report not mentioned). The isolated organism SB3 is similar to *Bacillus mycoides* with 91% probability.

Synthesis of ZnO nanoparticles and its recovery using CTAB + Hexane

After exposure of reaction mixture of cell-free extract of *B. mycoides* and 1mM concentration of $ZnSO_4.7H_2O$ as precursor salt in the ratio 1:1 to sunlight for two successive days, white precipitate was obtained in the flask. This showed formation of ZnO nanoparticles. This also indicated that the enzyme required for the reduction of nanoparticles is produced extracellularly by *Bacillus mycoides*. The phase transfer of the aqueous nanoparticles takes place into the organic phase. When the two layers were separated out, the phase transfer was observed by the white coloration of the organic phase and a corresponding almost complete loss of color from the aqueous phase. Kumar *et al*. [18], used same phase transfer method for recovery of platinum nanoparticles using a mixture of 100ml of 2x 10^4M Octadecylamine (ODA) with 100ml of 50% hexane. They used ODA as it is a good capping agent for platinum nanoparticles. In present study, CTAB is used as a capping agent as it acts as a good capping agent for Zinc nanoparticles [21]. After separating these nanoparticles were analyzed by UV- VIS spectroscopy for the range of 200-600 nm. Drying out the organic phase gave white color, fine dry powder which was stored into air tight tube and for further use.

Characterization of ZnO nanoparticles

The presence of ZnO nanoparticles was confirmed using double beam UV-Visible spectrophotometry. The absorption spectra of ZnO nanoparticles (Fig 1) exhibits a strong and broad absorption at approximately 380 nm. Maximum absorption for ZnO nanoparticles obtained by Zhiguo *et al*. [22] and Whagmare *et al*. [23] is at 380nm which is similar to that of present study. Mishra *et al*. [24] exhibited maximum absorption at approximately 315nm. Studies of Whagmare *et al*. [23] and Abdo *et al*. [25] revealed that the absorbance values peak lie between 300-380 nm. Thus, the study supports formation of ZnO nanoparticles.

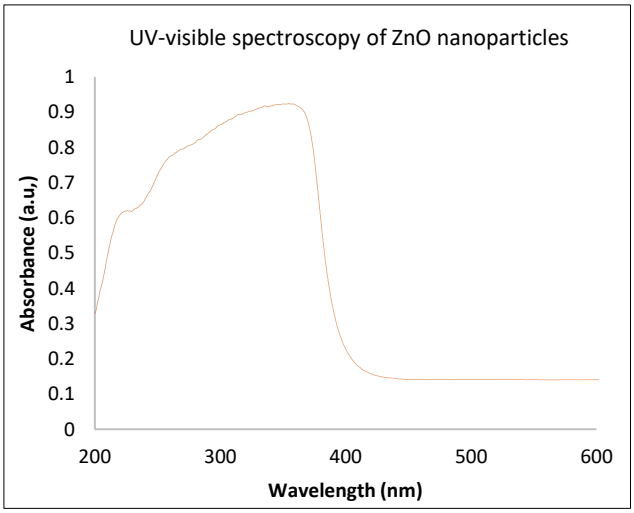

Fig 1 UV-Visible absorption spectrum for ZnO nanoparticles

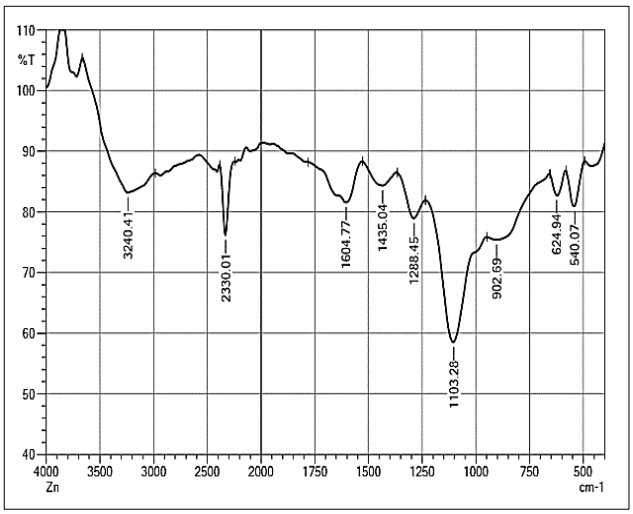

Fig 2 FTIR spectra of powdered ZnO nanoparticles

FTIR measurements of the powdered ZnO nanoparticles was carried out to identify the possible interactions between ZnO and bioactive molecules which may be responsible for synthesis and stabilization of ZnO nanoparticles. The FTIR spectrum shown of ZnO in (Fig 2) was operated within the spectrum range of 4000-400cm^{-1}. The bands at 3240.41cm^{-1} is due to stretching vibrations of O-H hydroxyl group and 1604.77 were assigned to the stretching vibrations due to C=C bond,

band at 1435.04cm^{-1} and 1238cm^{-1} was because of C-N amine stretching, indicating protein moiety to be responsible for stabilization of ZnO nanoparticles. C-O stretching lead to appearance of 1103.28cm^{-1} peak. The presence of atmospheric CO_2 gave band at 2330cm^{-1}. These results are similar to that of Saied *et al*. [26] (2013) having the peaks at 3396.9cm^{-1}for O-H stretching, band stretch of C=C and C-O at 1645cm^{-1} and 1028cm^{-1} respectively, which were closer to the bands obtained in present study. The range for the ZnO nanoparticle lies between 400-600 cm^{-1} [27]. Pattern of adsorption at 540.07cm^{-1} corresponds to Zn-O bonding [28-29], was also observed by Gnanasangeetha *et al*. (2013) and Geetha M.S. et al (2016). This, proves the presence of ZnO nanoparticles.

Analysis of XRD spectra depicted good crystalline structure of ZnO, nanoparticles. The presence of (100), (002), (101), (102), (110) and (103) planes (Fig 3) correspond with the well-defined peaks at 2θ values of 32.05°, 34.69°, 36.53°, 48.38°, 57.56°and 63.41° respectively, which was verified with JCPDS card (No. 5-0664). Size of nanoparticles was determined from FWHM, is in the range of 30~80nm. The average grain size (Z-average) of nanoparticle, calculated using Scherrer equation was 59.0nm [30].

The figure to the left indicates the SEM image for ZnO nanoparticles which is of 100µm resolution. The figure to the right represents the histogram of particle size distribution with 55nm particle size to be high in number). The Conformation of ZnO Nanoparticle morphology comes from the analysis of Scanning Electron Microscopy (SEM). The SEM micrograph along with histogram of particle size diameter in nm (Fig 4), shows individual ZnO nanoparticles as well as few aggregates. Form image and histogram, it is clear that the biosynthesized nanoparticles are spherical with particle size in the range of 30 ~70 nm with 55 nm size particles more in number. This result is in good agreement with that of values calculated from the X-ray diffraction. Similar shape of nanoparticles is reported by [20] with the size ranging in between 20-30nm.

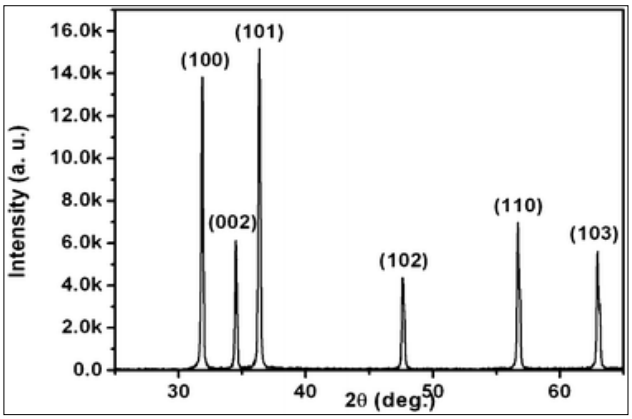

Fig 3 XRD pattern of ZnO nanoparticles

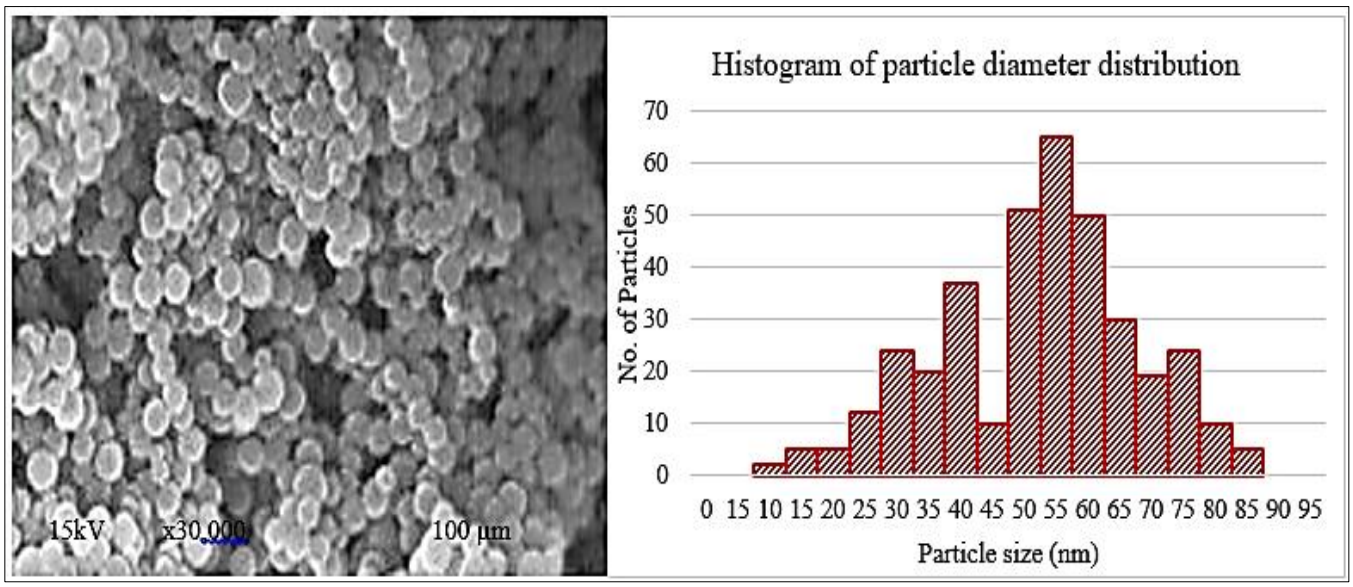

Fig 4 SEM images along with histogram of particle size diameter of ZnO nanoparticles

CONCLUSION

Twenty-three bacterial isolates were obtained from four zinc contaminated soil samples. Out of twenty-three isolates, isolate SB3 showed maximum tolerable metal concentration of 1000µg/mL. Isolate SB3 was Gram positive, non-motile, sub-terminal spore forming rod shape bacteria producing a specific rhizoidal flat colony. After biochemical and VITEK 2 characterization, isolate SB3 identified as *Bacillus mycoides*. Extracellular synthesis of ZnO nanoparticles using *Bacillus mycoides* was done successfully and recovered efficiently using phase transfer technique. These nanoparticles were characterized using UV-VIS spectroscopy, FTIR, XRD and SEM. These nanoparticles were found to be spherical in morphology with Z- average to be 55nm. From the above research, metal tolerant bacteria can be undoubtedly used for biological synthesis of nanoparticles. Further research will be done on the exploring the applications of ZnO nanoparticles in the field of medicine and waste water treatment.

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