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Biosynthesis, optimization and characterization of ZnO nanoparticles using *Bacillus cereus* MN181367 and their antimicrobial activity against multidrug resistant bacteria

Biosíntesis, optimización y caracterización de nanopartículas de ZnO usando *Bacillus cereus* MN181367 y su actividad antimicrobiana contra bacterias resistentes a múltiples fármacos

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Abstract

Zinc oxide nanoparticles (ZnO NPs) due to their unique properties have diverse applications in different fields of life. Bacterial synthesis of ZnO NPs is an eco-friendly, simple and inexpensive way. In this study, among eighteen bacterial isolates, eight confirmed ZnO NPs synthesis. On the basis of sharp absorption peak at 354 nm, growth conditions for gram positive *Bacillus cereus* H-SC1 were further optimized. Under different optimum parameters such as incubation temperature 37ºC, pH 9, inorganic salt $(NH_2)2SO_4$, SDS as surfactant, substrate $(ZnSO_4{\cdot}7H_2O)$ concentration 0.01 M and reaction time of two days under light condition, the ZnO NPs obtained had sharp peak at 352 nm and wide band gap of 3.5 eV. FTIR spectra indicated presence of amines and carbonyl groups as stabilizing agents. The scanning electron micrograph showed irregular shaped ZnO NPs and Zeta sizer indicated size ranging from 58.77-63.3 nm with PDI of 0.529. ZnO NPs exhibited negative zeta potential -7.39 mV. The antimicrobial assay by well diffusion method showed direct relationship of antibacterial activity with concentration of nanoparticles against *Escherichia coli* BTCB201, *Staphylococcus aureus* BTCB203 and *Salmonella typhi* BTCB202. Conclusively, bio-transformed ZnO NPs have great potential as alternative to conventional antibiotics and as drug delivery tool.

Keywords: Zinc Oxide NPs, *Bacillus cereus*, SEM, FTIR, ZnO NPs antimicrobial activity.

Resumen

Las nanopartículas de óxido de zinc (ZnO NP) debido a sus propiedades únicas tienen diversas aplicaciones en diferentes campos de la vida. La síntesis bacteriana de ZnO NPs es una forma ecológica, simple y económica. En este estudio, entre dieciocho aislados bacterianos, ocho confirmaron la síntesis de ZnO NP. Sobre la base de un pico de absorción agudo a 354 nm, las condiciones de crecimiento para *Bacillus cereus* H-SC1 gram positivo se optimizaron aún más. Bajo diferentes parámetros óptimos, tales como temperatura de incubación 37ºC, pH 9, sal inorgánica (NH_2) $2SO_4$, SDS como tensioactivo, concentración de sustrato $(ZnSO_4{\cdot}7H_2O)$ 0.01 M y tiempo de reacción de dos días en condiciones de poca luz, los NP de ZnO obtenidos tuvieron un pico agudo a 352 nm y banda ancha de 3.5 eV. Los espectros de FTIR indicaron la presencia de aminas y grupos carbonilo como agentes estabilizantes. La micrografía electrónica de barrido mostró ZnO NPs de forma irregular y el tamaño de Zeta indicaba un tamaño que oscilaba entre 58,77 y 63,3 nm con un PDI de 0,529. Los NP de ZnO exhibieron potencial zeta negativo -7.39 mV. El ensayo antimicrobiano por el método de difusión de pozos mostró una relación directa de la actividad antibacteriana con la concentración de nanopartículas contra *Escherichia coli* BTCB201, *Staphylococcus aureus* BTCB203 y *Salmonella typhi* BTCB202. En conclusión, los NP de ZnO biotransformados tienen un gran potencial como alternativa a los antibióticos convencionales y como herramienta de administración de fármacos.

Palabras clave: NPs de óxido de zinc, *Bacillus cereus*, SEM, FTIR, actividad antimicrobiana de ZnO NPs.

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1 Introduction

Metals nanoparticles have numerous applications due to their unique properties. Chemical and physical modes of synthesis are widely reported, however, nanoparticles that are produced by microbes are superior as compared to those produced by other known ways. The microbial route of synthesis is environment friendly and usage of costly chemicals is reduced by this way (De Silva *et al*., 2020; Li *et al*., 2011; Taranath and Patil, 2016; Zheng *et al*., 2017). Nanoparticles that are synthesized by this way have shown high catalyst reactivity, large surface area as well as better interaction between enzyme and the metal salt (Bhattacharya and Mukherjee, 2008). The extracellular biosynthesis of zinc oxide nanoparticles occured outside the bacterial cell. The mechanism entailed secretion of reduction enzymes like NADPH dependant reductase by bacteria in the supernatant solution of bacterial culture. The NADPH dependant reductase initiated reduction by transfer of electrons from NADPH to form $NADP^+$. The resulting electrons are attained by zinc ions that are present on the outside surface of cell and these ions are reduced to form elemental zinc oxide nanoparticles (Li *et al*., 2011; Zhang *et al*., 2011). The extracellular method of biosynthesis of nanoparticles is given preference over intracellular method beacuse it is cheap and devoid of the process of complex downstreaming (Ovais *et al*., 2018).

The inorganic zinc oxide nanoparticles have peculiar semi-conducting, photocatalytic, electrical, antibacterial, dermatological and optical properties (El Filali *et al*., 2015; Ovando-Medina *et al*., 2018; Wang, 2008). As compared to other nanoparticles, zinc oxide nanoparticles are inexpensive and have less toxicity; therefore, they have many applications (Kim *et al*., 2017). The ZnO NPs which have size less than 100 nm are considered biocompatible and suitable for biomedical applications (Jiang *et al*., 2018). In biomedical field, these NPs have applications like anti-cancer, anti-bacterial, anti-diabetic, anti-inflammation, healing of wound, drug delivery, bio-sensors and bio-imaging (Zhang and Xiong, 2015; Kim *et al*., 2017). Zinc oxide nanoparticles are not harmful to normal body cells of humans at concentration of upto 100 ug/ml and these can also be used as an alternative to antibiotics (Siddiqi *et al*., 2018). ZnO NPs are graded by US Food and Drug Administration (FDA) as generally recognised as safe (GRAS) (Jiang *et al*., 2018). Zinc oxide nanoparticles are non-toxic and easily diffuse in the food and prevent bacterial growth that is why these are used as food additives, preservatives in food packaging, and also preserve colors. Reportedly, there are no adverse effects of ZnO NPs on humans as daily requirment of zinc in human body is 10-15 mg and human body conatains 2-3 g of zinc (Siddiqi *et al*., 2018; Xie *et al*., 2011). In agricultural field, ZnO NPs can be effectively used as fungicide (He *et al*., 2011). In field of cosmetics because of UV-blockage properties of zinc oxide nanoparticles, they are used frequently in products of personal care such as sunscreens and cosmetics (Newman *et al*., 2009). ZnO NPs are dermally safe to use upto 1000 mg/kg body weight (Ryu *et al*., 2014). In textile industry, ZnO NPs are used in order to provide anti-bacterial properties and UV-absorbing properties to the textile fabrics (Wang *et al*., 2005).

The biosynthesis of ZnO NPs was reported by using *Acinetobacter schindleri* SIZ7, *Aeromonas hydrophila*, *Rhodococcus pyridinovorans* and *Aspergillus niger* (Jayaseelan *et al*., 2012; Kundu *et al*., 2014; Busi *et al*. 2016; Ibrahem *et al*., 2017). Zinc oxide nanoparticles of 68.41 nm size were reported to be synthesized by *Lactobacillus salivarius* (Salman *et al*., 2018). Using Candida albicans, biosynthesis of 20 nm sized ZnO NPs was done (Shamsuzzaman *et al*., 2017). The inadverent and long-term exposure to ZnO NPs can damage vulenarable human cells. Reportedly, the concentration dependent cytoxicity was examined and ZnO NPs of 50 nm size at concentration of 100 ug/ml reduced cell viability of human lung cells (Sahu *et al*., 2013). The mechanism proposed for anti-bacterial activity is generation of reactive oxygen species (ROS) like hydrogen peroxide that is very strong oxidizing agent and it causes harm to microbial cell (Sawai, 2003). On increasing the dose of particle, time of treatment and synthesis method, the nanoparticles become more effective (Dobrucka and Dlugaszewska, 2016). Zinc oxide nanoparticles with average size of 30 nm cause bacterial cell death (Jiang *et al*., 2018). ZnO NPs can stop growth of Gram positive as well as Gram negative bacteria (Fernando *et al*., 2018). ZnO NPs of 13 nm size were reported to inhibit *Escherichia coli* and *Staphylococcus aureus* growth at concentrations of 3.4 mM and >1 mM, respectively (Reddy *et al*., 2007). The antibacterial activity of ZnO NPs was reported against *Pseudomonas aeruginosa*, *Aspergillus flavus*, *Staphylococcus aureus*, *Bacillus subtilis* and *Campylobacter jejuni* (Xie *et al*., 2011; Jayaseelan *et al*., 2012; Lakshmi *et al*., 2012; Ibrahem *et al*., 2017). The present study was intended towards bacterial synthesis of ZnO NPs which is an eco-friendly method having antimicrobial potential against multidrug resistant pathogens.

2 Materials and methods

2.1 Sample collection and isolation

The soil sample was collected from Lahore College for Women University, Pakistan. Different isolates of bacteria were isolated by serial dilution method from soil sample (Karadayi *et al*., 2017).

2.2 Biosynthesis of ZnO nanoparticles

For biosynthesis of ZnO NPs, inoculation of each bacterial isolate was done in 500 ml conical flask containing 100 ml of autoclaved nutrient broth and then incubation was done at 37°C for 24hrs in shaking incubator (IRMECO, Germany) at 121 rpm. After incubation, bacterial growth was measured at 600 nm using UV 1100 Spectrophotometer (Robus technologies, UK). Culture was centrifuged at 6500 rpm for 20 minutes and supernatant was saved for further processing whereas pellet was discarded. For bio-reduction of metal, zinc sulphate hepta-hydrate solution (0.01 M) was mixed with cell free extract in 1:1 ratio. The mixture was incubated at 37°C, 121 rpm in shaking incubator (IRMECO, Germany) for 48 hrs. After 2 days of incubation, colour change and UV-absorbance (200-1000 nm) of reaction mixture was observed (Busi *et al*., 2016).

2.3 Optimization of different parameters

Based upon the colour, duration and excitation peak of ZnO NPs single isolates was selected and further optimized at different physicochemical conditions. The different parameters optimized were: Incubation temperatures ranging from 30ºC, 37ºC, 40ºC, 45ºC and 50ºC (Nagarajan and Kuppusamy, 2013; Sundaraselvan and Quine, 2017; Gupta *et al*., 2018). pH ranging from 5, 6, 7, 8 and 9 (Nagarajan and Kuppusamy, 2013; Gupta *et al*., 2018; Jamdagni *et al*., 2018; Mohammadi and Ghasemi, 2018). Inorganic salts of 1 mM concentration of each Magnesium sulphate ($MgSO_4$), sodium chloride (NaCl), copper sulphate ($CuSO_4$), ammonium sulphate ($(NH_4)_2SO_4$) and monopotassium phosphate (KH_2PO_4) was used (Bae *et al*., 2002; Sastry *et al*., 2013). Similarly, different surfactants sodium dodecyl sulphate (SDS), ethylenediamine tetraacetic acid (EDTA), Tween 80, Tween 20, and polyethylene glycol (PEG) of 1 mM concentration were used. Different substrate concentration of $ZnSO_4 \cdot 7H_2O$ i.e. 0.005 M, 0.05 M, 0.001M, 0.01 M and 0.1 M and incubation time i.e. 0 minutes, 30 minutes, 1 hour, 2 hours, 1 day and 2 days along with light and dark conditions were also observed (Dalai *et al*., 2012; Morsy, 2014).

2.4 Molecular identification of isolate of bacteria by 16S rDNA sequencing

On the basis of 16S rDNA sequence, identification of bacterial isolate H-SC1 was done through First Base Company (Singapore) using sequencing primers 785F 5' (GGA TTA GAT ACC CTG GTA) 3' and 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3' (Hasan *et al*., 2019). PCR primers used were 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'. The phylogenetic tree was constructed using Mega 5.05 software (Busi *et al*., 2016; Kumar *et al*. 2008). The isolate H-SC1 was identified as *Bacillus cereus* (NCBI Genbank Accession No. MN181367).

2.5 Characterization of ZnO NPs

The characterization of biosynthesized ZnO NPs was done by FTIR spectrometer IRTracer-100 (SHIMADZU, NA), Scanning electron microscope EVO LS 10 (ZEISS, USA) and Zeta sizer Nano Range (Malvern, UK). FTIR was used to find the functional groups attached to ZnO NPs (Busi *et al*., 2016). Topological information of the ZnO NPs was done by scanning electron microscope (model) (Datta *et al*., 2017). Zeta sizer gave information about the size and potential of the of ZnO NPs (Kavitha *et al*., 2017).

2.6 Anti-bacterial activity of zinc oxide nanoparticles against multi-drug resistant bacteria

The antimicrobial activity of bio-synthesized zinc oxide nanoparticles against multidrug resistant bacteria like *Escherichia coli* BTCB201, *Staphylococcus aureus* BTCB203 and *Salmonella typhi* BTCB202 was tested by agar well diffusion method (Jaidev and Narasimha, 2010). Inoculum of pathogen used was set according to 0.5 Mcfarland standard (McFarland, 1907). Different concentrations of the ZnO NPs 0.1 ug/ml to 0.6 ug/ml were used to study the antibacterial activity (Busi *et al*., 2016).

2.7 *Statistical analysis*

All the experiments were carried out in triplicates. One way ANOVA was used for finding out the significant difference ($p \leq 0.05$) between means of different parameters by SPSS program along with application of Duncan's multiple range test (Duncan, 1955).

3 Results and discussion

3.1 *Biosynthesis of ZnO NPs*

The synthesis of ZnO NPs by *Bacillus cereus* MN181367 was indicated by colour change from pale yellow to fluorescent yellow (Fig. 1-a). The synthesis of ZnO NPs was further confirmed by its UV-Vis spectrum which showed sharp peak at minimum wavelength of 354 nm with maximum absorbance of 1.772 (Fig. 1-c). The UV-Vis spectrophotometer analysis of all other 18 isolates was also done (Fig. 1-b). The absorption peaks within wavelength of 350 nm-380 nm indicated synthesis of nano-sized ZnO particles. Nano-sized ZnO particles upon synthesis showed increase in band gap and the absorption spectrum shifts towards the lower wavelength (Singh *et al*., 2019). ZnO NPs of bulk size were reported in other studies with absorption peaks of higher wavelengths in the range of 360 nm-381 nm (Awwad *et al*., 2020; Jayaseelan *et al*., 2012; Shamsuzzaman *et al*., 2017).

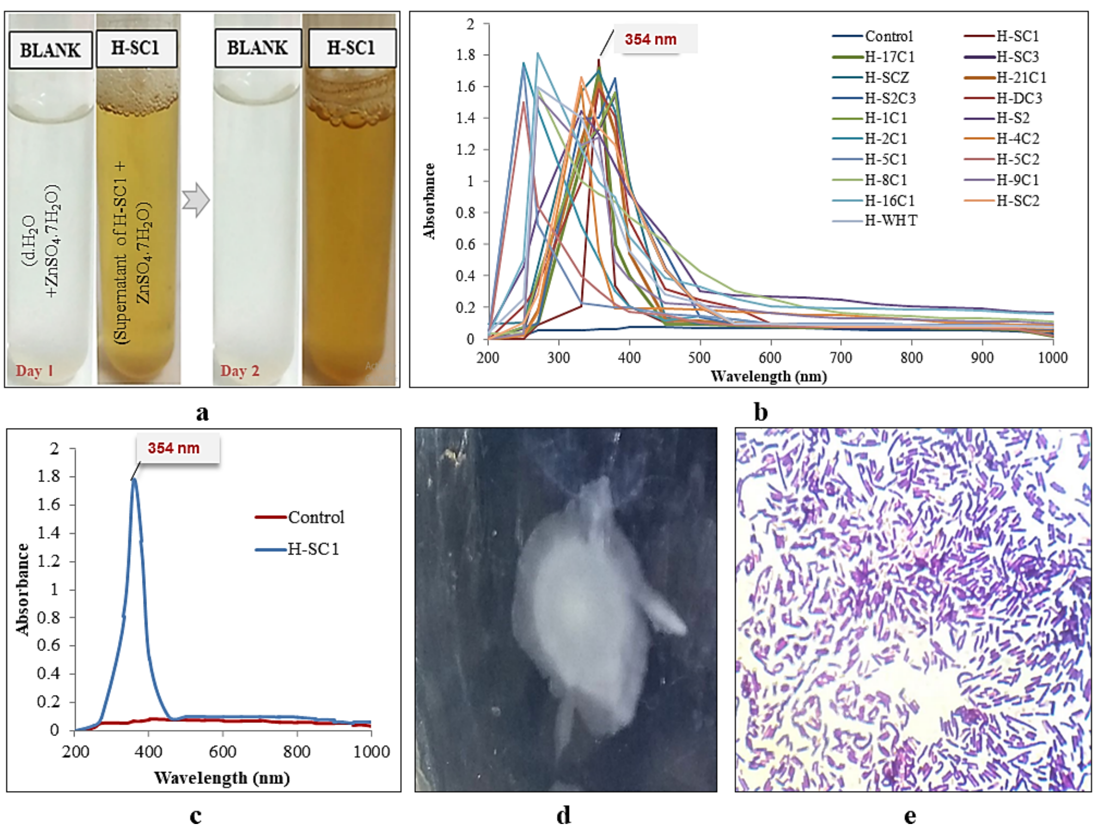

Fig. 1 (a) Bacterial isolate H-SC1 producing fluorescent yellow colour indicating synthesis of ZnO NPs; (b) UV-Vis spectrophotometer analysis of all bacterial isolates; (c) UV-Vis spectrophotometer analysis of bacterial isolate H-SC1 synthesizing ZnO NPs; (d) Colonial morphology of bacterial isolate H-SC1; (e) Gram positive rods of *Bacillus cereus* H-SC1 at 100X oil immersion.

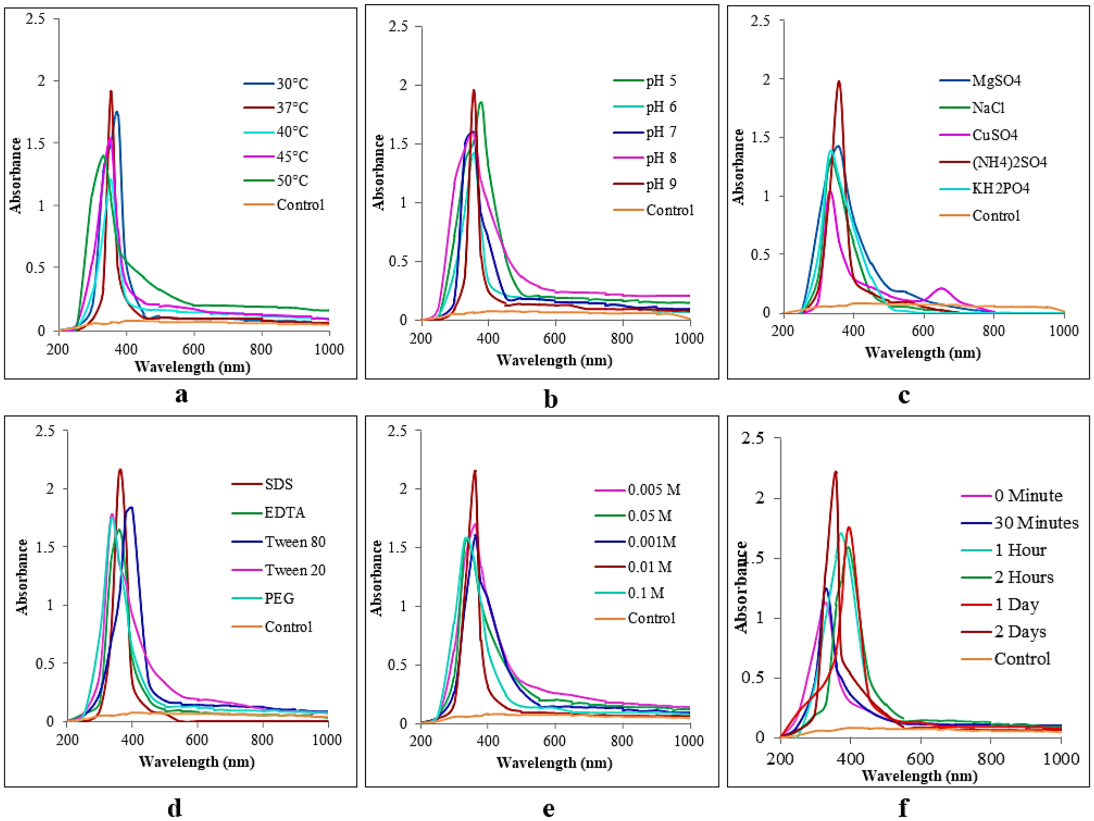

Fig. 2 (a) UV-Vis spectrophotometer analysis of biosynthesized ZnO NPs from *Bacillus cereus* MN181367 under different incubation temperatures; (b) UV-Vis spectrophotometer analysis of biosynthesized ZnO NPs from *Bacillus cereus* MN181367 at different pH ranges; (c) UV-Vis spectrophotometer analysis of biosynthesized ZnO NPs from *Bacillus cereus* MN181367 by different inorganic salts; (d) UV-Vis spectrophotometer analysis of biosynthesized ZnO NPs from *Bacillus cereus* MN181367 by using different surfactants; (e) UV-Vis spectrophotometer analysis of biosynthesized ZnO NPs from *Bacillus cereus* MN181367 at different substrate concentrations; (f) UV-Vis spectrophotometer analysis of biosynthesized ZnO NPs from *Bacillus cereus* MN181367 at different reaction times.

3.2 Effect of incubation temperature

The optimum incubation temperature for ZnO NPs synthesis by *Bacillus cereus* MN181367 was selected to be 37ºC because of sharp absorption peak with maximum absorbance of 1.92 at lower wavelength of 354 nm which indicated nano-sized ZnO particles (Fig. 2-a). The high temperature led to high reaction kinetics which caused increased reduction of metal ions into elemental nanoparticles (Nagarajan and Kuppusamy, 2013; Mohammadi and Ghasemi, 2018; Yusof *et al*., 2019). Temperature has effect on bacterial growth varying the levels of enzymes and thereby affects nanoparticles synthesis, as mesophilic microbes do not tolerate high temperatures therefore by them nanoparticles synthesis at elevated temperature is not suitable (Roopan *et al*., 2013). In some studies synthesis of ZnO NPs was observed at different temperatures from 25 ºC to 90 ºC and gave absorption peaks in the range of 355-368 nm (Gupta *et al*., 2018; Jamdagni *et al*., 2018; Yusof *et al*., 2019).

3.3 Effect of pH

In this study, *Bacillus cereus* MN181367 at pH 9 gave sharper peak with maximum absorbance (1.956) at the lower wavelength of 354 nm (Fig. 2-b). The higher pH caused increase in reduction of metal ions to form nanoparticles. The pH actually altered the electrical charges of biomolecules and these biomolecules changed the reducing as well as capping ability and affected synthesis of nanoparticles. The increase in pH from 4 to 8 gave absorption peaks at higher wavelengths (red shift) indicating synthesis of large sized nanoparticles while increase in pH from 8-10 indicated blue shift (lower wavelength) of absorption peaks and synthesized small sized nanoparticles (Nagarajan and Kuppusamy, 2013; Mohammadi and Ghasemi, 2018). Synthesis of ZnO NPs was observed from pH 4 to pH 14 in other studies giving absorption peaks in the range of 365nm-373 nm (Gupta *et al*., 2018; Mohammadi and Ghasemi, 2018).

3.4 Effect of inorganic salts

The salt $(NH_4)_2SO_4$ was considered optimum for ZnO NPs synthesis because it gave sharper absorption peak with maximum absorbance of 1.981 at the lower wavelength of 354 nm (Fig. 2-c). The metal ions addition caused increase of growth and production of enzyme which resulted in reduced size of nanoparticles. Genomic and proteomic responses are generated by microbes in regulation of metal homeostasis and results in attachment of heavy metals to cell by membrane proteins in reaction mixture that results into NPs synthesis (Sintubin *et al*., 2009; Schluter *et al*., 2014).

3.5 Effect of surfactants

The surfactant SDS was considered suitable for ZnO NPs synthesis because it gave sharper absorption peak with maximum absorbance of 2.149 at the lower wavelengths of 352 (Fig. 2-d). Surfactants form an absorption layer on nanoparticles and stops aggregation of particles by increasing repulsion forces between these particles. SDS was selected because it causes the large production of small sized stable ZnO NPs. However in another study uniform ZnO NPs were synthesized in the presence of polyethylene glycol 2000 (Morsy, 2014).

3.6 Effect of substrate ($ZnSO_4 \cdot 7H_2O$) concentration

The substrate concentration of 0.01 M was considered optimum for zinc oxide nanoparticles synthesis because of sharper peak at smaller wavelength of 352 nm and maximum absorption of 2.151 (Fig. 2-e). As the concentration of Zn^{+2} increased, it caused increase in absorption and shaper peaks were obtained and growth of nanoparticles was also enhanced but if concentration of substrate is increased beyond threshold value then it caused broadening of peaks and less absorbance and reduced ZnO NPs synthesis (Mohammadi and Ghasemi, 2018). In other studies 0.1 M zinc sulphate was considered optimum where absorption peaks were obtained at 356 nm and 373 nm (El Waseif *et al*., 2017; El Waseif, 2019).

3.7 Effect of incubation time

Incubation time of 2 days was considered optimum as sharp peak was obtained 352 nm, with maximum absorption of 2.201 (Fig. 2-f). The increase of reaction time showed increased formation of ZnO NPs because of metal ions conversion to elemental nanoparticles (Gupta *et al*., 2018). The optimum time studied for ZnO NPs synthesis was reported to be 3-2 hours to 2 days in other studies giving absorption peaks at wavelength range of 363 nm-365 nm (Kalaiselvi *et al*., 2016; Gupta *et al*., 2018).

3.8 Effect of light and dark condition

The light condition for ZnO NPs biosynthesis was considered optimum as UV-Vis spectrum showed sharper absorption peak at 352 nm with wide band gap of 3.5 eV (Fig. 3-a). The band gap was increased and as result absorption peak was obtained at lower wavelength and reduced size of ZnO NPs (Singh *et al*., 2019).

3.9 Bacterial identification

The colony morphology of *Bacillus cereus* MN181367 was observed to be opaque, large sized, flat, irregular shaped and white pigmented (Fig. 1-d). The Gram staining showed Gram positive rods (Fig. 1-e). The molecular identification indicated bacterial isolate H-SC1 identified as *Bacillus cereus* as it showed 99% similarity with *Bacillus cereus* (Fig. 3-c).

3.10 Fourier transform infrared spectroscopy (FTIR)

FTIR spectrum of $ZnSO_4.7H_2O$ (control) showed the peaks at wavenumbers of 501.49 cm^{-1} (C-I stretching), 601.79 cm^{-1} (C-Br stretching), 648.08 cm^{-1} (C-Br stretching), 759.95 cm^{-1} (C-Cl stretching), 825.53 cm^{-1} (C-Cl stretching), 864.11 cm^{-1} (C-H bending), 887.26 cm^{-1} (C=C bending), 1091.71 cm^{-1} (C-O stretching), 1288.45 cm^{-1} (C-O stretching) and 1334.74 cm^{-1} (O-H bending), 1419.61 cm^{-1} (O-H bending), 1635.64 cm^{-1} (C=C stretching), 3005.10 cm^{-1} (C-H stretching) and 3332.99 cm^{-1} (N-H stretching) (Fig. 4-a). After bio-reduction, ZnO NPs were synthesized and their FTIR spectra showed peaks at the wavenumbers of 667.37 cm^{-1}, 1635.64

cm^{-1}, 2360.87 cm^{-1}, 3336.85 cm^{-1} and 3996.51 cm^{-1} which corresponded to presence of different functional groups H-Br stretching (alkyl halides class), C=C stretching (conjugated alkene), O=C=O stretching (compound class of carbon dioxide), N-H stretching (secondary amines) and O-H stretching (class of alcohol), respectively. The absorption peaks in the range between 400 cm^{-1} to 600 cm^{-1} were assigned as zinc oxide nanoparticles. The absorption bands in this FTIR spectrum at 482.20 cm^{-1}, 513.07 cm^{-1} and 597.93 cm^{-1} were particularly assigned as the stretching vibrations of ZnO NPs.

The comparison of FTIR spectra of control and ZnO NPs was done, it was observed that after bio-reduction peaks from 756.95 cm^{-1} to 1419.61 cm^{-1} were removed indicating removal of different functional groups and on the other hand, peaks at 2360.87 cm^{-1} and 3996.51 cm^{-1} appeared indicating class of carbon dioxide and alcohol group added. It was observed that biosynthesized ZnO NPs have more stability for a very longer time without causing any agglomeration due to presence of different biomolecules and proteins on their surface (Ovais *et al*., 2018). The alcoholic groups have capability of binding with metals therefore promoting capping and stability and stopping agglomeration. FTIR spectra of ZnO NPs, in other studies, showed Zn-O stretches at 466.77 cm^{-1}, 482 cm^{-1}, 513 cm^{-1}, 515 cm^{-1}, 584 cm^{-1} and 612 cm^{-1} (Awwad *et al*., 2020; Dobrucka and Dlugaszewska, 2016; Maruthupandy *et al*., 2016; Kavitha *et al*., 2017). The peaks at 3479.58 cm^{-1}, 1656.36 cm^{-1} and 1750 cm^{-1} referred to presence of O-H stretch (hydroxyl group of alcohols), C=C stretch (aromatic alkenes) and C=O stretch (carboxylic acid), respectively (Maruthupandy *et al*., 2016; Kavitha *et al*., 2017).

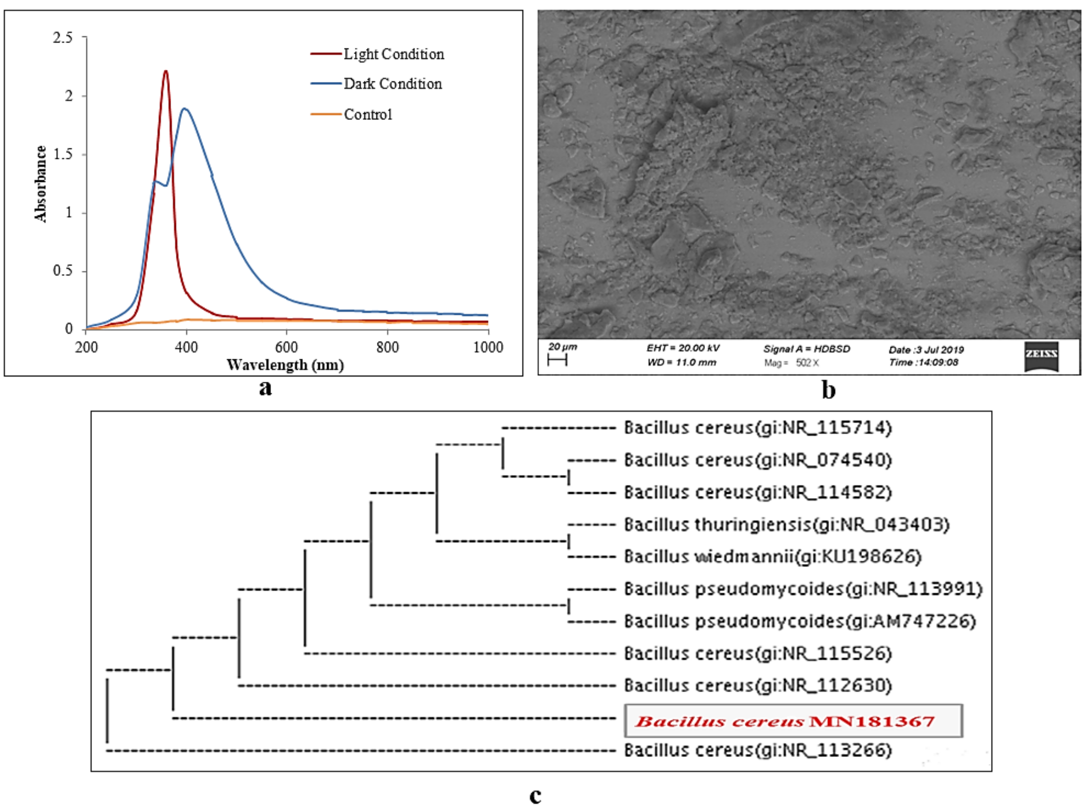

Fig. 3 (a) UV-Vis spectrophotometer analysis of biosynthesized ZnO NPs from Bacillus cereus MN181367 under light and dark conditions; (b) SEM of ZnO NPs biosynthesized by Bacillus cereus MN181367 appeared to be irregular shaped; (c) Phylogenetic tree of *Bacillus cereus* MN181367.

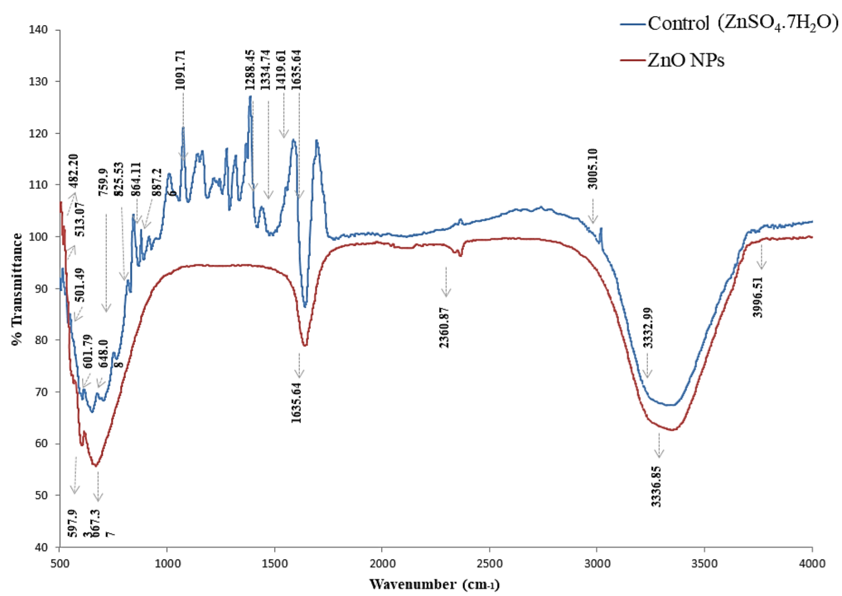

Fig. 4 FTIR spectrum indicating comparison of functional groups attached to ZnO NPs and $ZnSO_4{\cdot}7H_2O$.

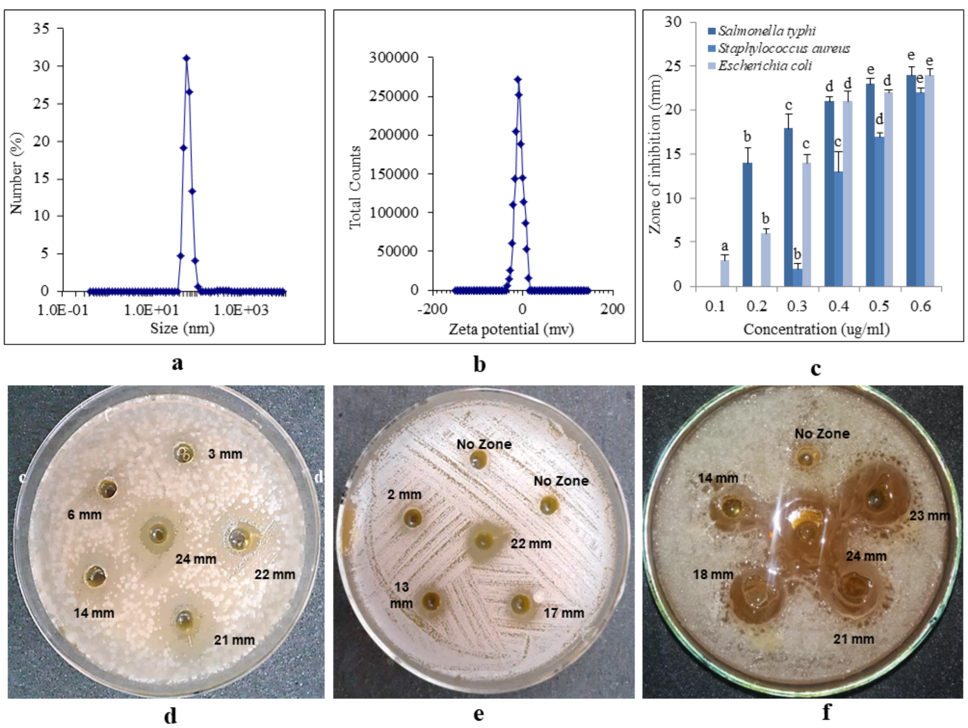

Fig. 5 (a) Zeta size of ZnO NPs biosynthesized by Bacillus cereus MN181367; (b) Zeta potential of ZnO NPs biosynthesized by *Bacillus cereus* MN181367; (c) Antimicrobial activity of ZnO NPs against *Escherichia coli* BTCB201, *Staphylococcus aureus* BTCB203 and *Salmonella typhi* BTCB202. Each value is the mean obtained from three triplicates. Superscripts on bars indicated significant difference at $p \leq 0.05$ by Duncan's Multiple Range Test; (d) Zones of inhibition produced by ZnO NPs against *Escherichia coli* BTCB201; (e) Zones of inhibition produced by ZnO NPs against *Staphylococcus aureus* BTCB203; (f) Zones of inhibition produced by ZnO NPs against *Salmonella typhi* BTCB202.

3.11 Scanning electron microscope (SEM)

The biosynthesized ZnO NPs were observed in scanning electron microscope. SEM results indicated that they were irregular shaped (Fig. 3 b). While in another study ZnO NPs were reported to be like nano-wires, spheroidal, rod shaped, geometrical shaped, irregular shaped and nano-rods (Hassan *et al*., 2020; Lopez-Cuenca *et al*., 2019; Umamaheswari *et al*., 2018).

3.12 Zeta sizer

Zeta sizer indicated size of ZnO NPs to be 58.77-63.3 nm with PDI of 0.529 with 99.65% intensity (Fig. 5-a). Results indicated nano-sized and monodispersed ZnO particles. Reportedly, the PDI value less than 0.7 is reported to be indication for monodispersed nanoparticles (Umar *et al*., 2019). Sizes of ZnO NPs were reported as 112.87 nm and 82.31 nm and PDI values were 0.326 and 0.262 in other studies (Hayeemasae *et al*., 2018; Umar *et al*., 2019).

3.13 Zeta potential

The results indicated that ZnO NPs exhibited negative zeta potential 7.39 mV (Fig. 5-b). The magnitude of zeta potential value showed stability of nanoparticles. The negative zeta potential indicated that NPs have net negative charge on their surface. The value of zeta potential between -10 mV to +10 mV of nanoparticles is considered almost neutral (Clogston and Patri, 2011). Negative zeta potential of 23.92 mV was reported in other study which indicated higher stability of ZnO NPs (Abdelhakim *et al*., 2020).

3.14 Anti-bacterial activity of ZnO NPs against multidrug resistant bacteria

The antimicrobial activity of biosynthesized ZnO NPs was evaluated by measurement of diameter of inhibitory zones against three different pathogenic multidrug resistant bacteria i.e. *Escherichia coli* BTCB201, *Staphylococcus aureus* BTCB203 and *Salmonella typhi* BTCB202 (Fig. 5-c, Fig. 5-d, Fig. 5-e, Fig. 5-f). ZnO NPs at 0.6 ug/ml concentration showed 8-folds increase in antimicrobial activity as compared to 0.1 ug/ml concentration against *Escherichia coli* BTCB201. In case of *Staphylococcus aureus* BTCB203, the inhibitory zone obtained at 0.6 ug/ml concentration of ZnO NPs showed 11-folds increase as compared to inhibitory zone obtained at 0.3 ug/ml concentration while at 0.1 ug/ml and 0.2 ug/ml, no zone of inhibition was formed. ZnO NPs at concentration of 0.6 ug/ml showed inhibitory zone of 24 mm for *Salmonella typhi* BTCB202 that was 1.71 folds higher than the inhibitory zone (14 mm) obtained at concentration of 0.2 ug/ml while at 0.1 ug/ml, no zone was obtained. On the other hand, ZnO NPs at 0.6 ug/ml gave inhibitory zone of 24 mm against *Escherichia coli* BTCB201 and *Salmonella typhi* BTCB202 which was 1.09 folds higher than the inhibitory zone (22 mm) obtained against *Staphylococcus aureus* BTCB203. The results indicated that the inhibitory effect of zinc oxide nanoparticles increased with the increase of concentration. The sizes as well as the concentrations of ZnO nanoparticles are very significant factors in anti-microbial activity of ZnO NPs (Liu *et al*., 2009; Nilavukkarasi *et al*., 2020). Direct interaction of zinc oxide nanoparticles to the cell surface of bacteria causes cell membrane to become permeable and also triggers oxidative stress by inactivation of enzymes eventually causing death of the cell (Gupta *et al*., 2018). Reportedly, ZnO NPs showed antibacterial activity against Bacillus subtilis and *Escherichia coli* and with *Escherichia coli* gave maximum zone of inhibition of 15 mm and 16 mm (Meruvu *et al*., 2011; Mohammadi and Ghasemi, 2018).

Conclusion

Conclusively, bacterially synthesized ZnO nanoparticles from *Bacillus cereus* MN181367 have potential as antimicrobial agent against multidrug resistant pathogens at very low concentration and therefore have promising future.

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