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Antibacterial activity and characterization of zinc oxide nanoparticles synthesized by microalgae

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Abstract: Biosynthesis of zinc oxide nanoparticles (ZnO-NPs) using microalgae is novel and cost-effective approach. We studied production, molecular characterization, and antibacterial activity. Filtrates of isolated microalgae strain ZAA1 (MF140241), ZAA2 (MF114592) and ZAA3 (MF114594) were used. Incubation of these strains in 5mM solution of zinc nitrate was resulted in the synthesis of ZnO-NPs. Fourier-transform infrared, UV-visible spectroscopy and scanning electron microscopy were used to characterize the nanoparticles. Significant antibacterial activity of ZnO-NPs was measured against *Escherichia coli, Staphylococcus aureus, Micrococcus luteus, Klebsiella pneumoniae* and *Citrobacter freundii*. The microalgae mediated ZnO-NPs production is a successful procedure that can be used in a wide range of biomedical applications.

Keywords: ZnO nanoparticles, microalgae, molecular characterization.

INTRODUCTION

Nanoscience study the materials at sub-molecular, molecular and macromolecular level to synthesize nanoparticles for various scientific fields (Bayford *et al.*, 2017; Huang *et al.*, 2015). For nanoparticles synthesis, the synthetic techniques are more expensive, complex production mechanism, cytotoxicity issues, instability and have many challenges to fig. out (Nune *et al.*, 2009; Mohammadi-Samani and Taghipour, 2015). In this study, we used green method to synthesize zinc oxide nanoparticles (ZnO-NP) using zinc oxide that has a wide range of commercial applications (Oladiran and Olabisi, 2013; Bondarenko *et al.*, 2013). Synthesis of nanoparticles through biological means is preferred due to its diverse uses and numerous advantages over synthetic techniques. Microorganisms are one of the effective biological methods to reduce metal salts into detoxified metal nanoparticles (Singh *et al.*, 2016; Logeswari *et al.*, 2015). These naturally produced NPs are renewable, easily degradable and do not require any support systems and destructive chemical agents (Issaabadi *et al.*, 2017).

Algae are one of the important microbial species to synthesize NPs because of its vast strains. A number of biotechnological applications including health, medical, diagnostic agent, agriculture, biofuel production and environmental aspects are associated with algae and due to this importance (Mohammed, 2015), we focused to use algae for the synthesis of nanoparticles.

Until now, a variety of algal strains like *Chlorophyceae*, *Phaeophyceae*, Cyanophyceae, *Rhodophyceae* (diatoms and euglenoids) have been studied for NPs biosynthesis (Sharma *et al.*, 2015). The ability of algae to harvest metals and reduce metal ions makes them the appropriate candidate for NP synthesis. Algae, along with various other benefits such as low temperature synthesis and increased energy efficiency, less toxicity and environmental risks, are comparatively appropriate and simple to handle. In view of the use of the environmentally safe methods, the zinc oxide NPs were synthesized and the antimicrobial effects of ZnO - NPs were investigated. Therefore, this study investigates the potential application of algal-mediated synthesis of nanoparticles of biotechnological importance.

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MATERIALS AND METHODS

Isolation and culturing of microalgae strains

Microalgae cultures were isolated by means of knife, spatula and fitted substrata from fresh water samples of ponds, lakes, fields and aquariums in autoclaved bottles (Lee *et al.*, 2014) collected from Multan, Southern region of Punjab, Pakistan. All samples were labeled side by side. The samples were then stored under low light intensity in cool environments (Aliya *et al.*, 2009). 5ml of microalgae water samples were transferred to the autoclaved glass vial containing 250ml BG-11 modified medium with pH 7-9 (table 1). These glass bottles were covered by cotton plugs and kept under the sun at 15 to 23°C with continuous stirring (Wu *et al.*, 2013). The grown cultures were inoculated onto BG-11 agar media petri plates (15gm agar is dissolved in 1L of BG-11) and incubated under sunlight for 7-10 days. After a week different colony of microalgae were observed (Ogbonna and Ogbonna, 2015). These colonies were sub-cultured and purified by standard streak plate technique (Duong *et al.*, 2013; Promdaen *et al.*, 2014). The study was focused

to design ZnO-NPs using microalgae cultures with integrated framework (fig. 1).

Characterization of microalgae cultures

Morphological identification

Culture was morphologically identified by Olympus Compound microscope and digital images were accessed using a Nikon E5000 digital camera. Algae were cultivated in BG-11 liquid broth during 7 - 10 days for microscopic analysis. The number of cells was recorded per coenobium, cell configuration and shape (Kumar *et al.*, 2014).

DNA extraction, PCR, and sequence analysis

For DNA extraction, 10ml each of microalgae cultures were taken in falcon tubes, centrifuged at 4000rpm on 25°C for 20 minutes. Supernatant was removed and biomass was taken in pestle-mortar. Biomass washed with distillated water to prevent contamination and DNA from algal cultures was extracted using cetyl trimethyl ammonium bromide (CTAB). A suitable amount of CTAB-buffer (65°C) were added and continuously mixed to form a homogenized-paste. The CTAB-buffer contained 1M Tris, 5M NaCl, 10g CTAB, and 0.5M EDTA. We added proteinase K, chloroform isoamyl alcohol (CIA), β-mix, and polyvinyl-pyrrolidone (PVP) into mixed contents. DNA was isolated and kept overnight at 4°C before 5µL of the DNA was run on test gel (Wongsawad and Peerapornpisal, 2014). The genome was amplified using Agilent PCR thermocycler. The PCR-program was followed as: 95°C, 5min; 94°C, 45s (denaturation); 55°C, 30s (annealing); 72°C, 2min (DNA synthesis, elongation); repeated for 30 cycles; 72°C, 10min; and 4°C hold.

Sequencing of genome

In the sequencing, Big Dye, primers, sequencing buffer, and DNA were used. 16S rRNA was sequenced using primer pair: 106-F and 781-R, 106-R+781-F. The samples were sequenced according to the standard procedure. PRISM BigDye Terminator v3.1 Cycle Sequencing Kit was used for sequencing. In 30µL reaction mixture, genomic DNA was taken as a template with EF-Taq. For 2 minutes, taq polymerase was applied at 95°C. 35 cycles were completed at 95, 55 and 72°C for 1 minute. Finishing was done at 72°C for 10 minutes (Liu *et al*., 2013). Multi-screen filter paper was used for purification of replication (Manoylov, 2014). The extension of DNA products to Hi-Di formamide was then put in. At 95°C, cool the mixture on ice for five minutes and analyzed it in the ABI PRISM 3730XL DNA Analyzer (Jenaa *et al*., 2013; Patela *et al*., 2015).

Construction of phylogenetic tree

The sequences of microalgae strains were aligned with known sequencing data available at the NCBI database by Basic Local Alignment Search Tool (BLAST) and percentage of homology were recorded. Using online server, Clustal Omega (http://www.ebi.ae.ut/Tools/msa/clustalo/) was used to construct phylogenetic tree based on neighbor-joining method.

Synthesis of ZnO-NPs by microalgae

The monocultures of each microalgae strain were taken in 15ml falcon tubes. These tubes were centrifuged at 4500 rpm, at 20°C for 25 minutes and precipitated biomass was washed with 10ml of sterile distilled water (Azam *et al.*, 2012). The culture was lysed by keeping in water bath at 100°C for one-hour. The sample tubes were centrifuged and microalgae extract was retained (Osman and Mustafa, 2015). For preparation of zinc oxide nanoparticles, 20ml of 5mM sterile solution of zinc nitrate was added in 5ml of microalgae extract (Azam *et al.*, 2012) and incubated for 36 to 40 hours at 30°C. The reaction tubes after centrifugation at 10°C for 5 minutes at 6000 rpm showed a cloudy appearance in microalgae extracts (Osman and Mustafa, 2015).

$Zn(NO_3)_2$ + Microalgae extract → ZnO-NPs + Byproducts

Antibacterial activity of ZnO-NPs

Antibacterial activity of ZnO-NPs was tested against *Escherichia coli*, *Staphylococcus aureus, Micrococcus luteus, Klebsiella pneumoniae and Citrobacter freundii* by standard well diffusion assay. All the bacterial strains were cultivated for 24 hours in nutrient broth at 37°C and McFarland standard was maintained in the bacterial cell density. The inoculum of these indicator cultures was uniformly spread onto nutrient agar plate and 60µL of ZnO-NPs sample solution and ciprofloxacin as standard antibiotic were poured into each well of the plate. These petri plates were incubated for 24 hours at 37°C. We observed the zone of inhibitions in terms of millimeter scale (Chang and Tsai, 2008).

Characterization of ZnO-NPs

Scanning Electron Microscopy (SEM)

The SEM was performed to characterize the morphological features and sample's surface topography of ZnO-NPs (Talam *et al.*, 2012; Geetha *et al.*, 2016).

UV-Vis spectroscopy

The absorbance and optical properties of ZnO-NPs was assessed by using UV-Vis spectrophotometer. The absorbance at 358nm indicated the mono-dispersed nature of particles (Chang and Tsai, 2008; Manivasagan and Kim, 2015).

Fourier Transform Infrared spectroscopy (FTIR)

In order to observe the functional groups of ZnO-NPs, FT-IR was performed. Freeze dried sample fractions were used and spectral plot was obtained at 400-4000 cm^{-1} (Oscar *et al.*, 2016).

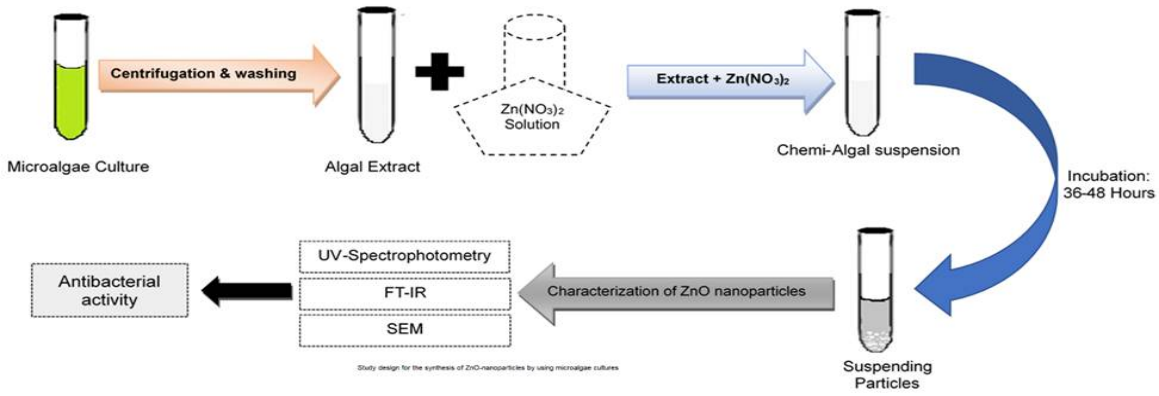

Fig. 1: Production, characterization and antibacterial activity of ZnO-NPs by microalgae

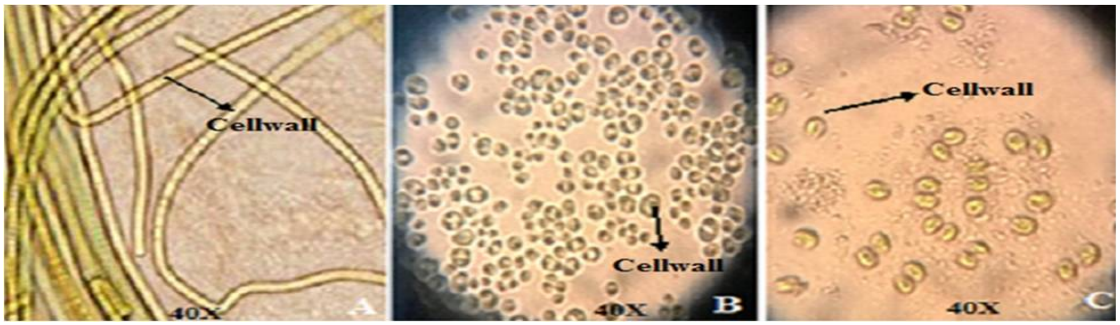

Fig. 2: (A) Thread like filamentous green microalgae ZAA1 strain (B) Spherical coccid microalgae ZAA2 (C) Round centrally distorted microalgae ZAA3

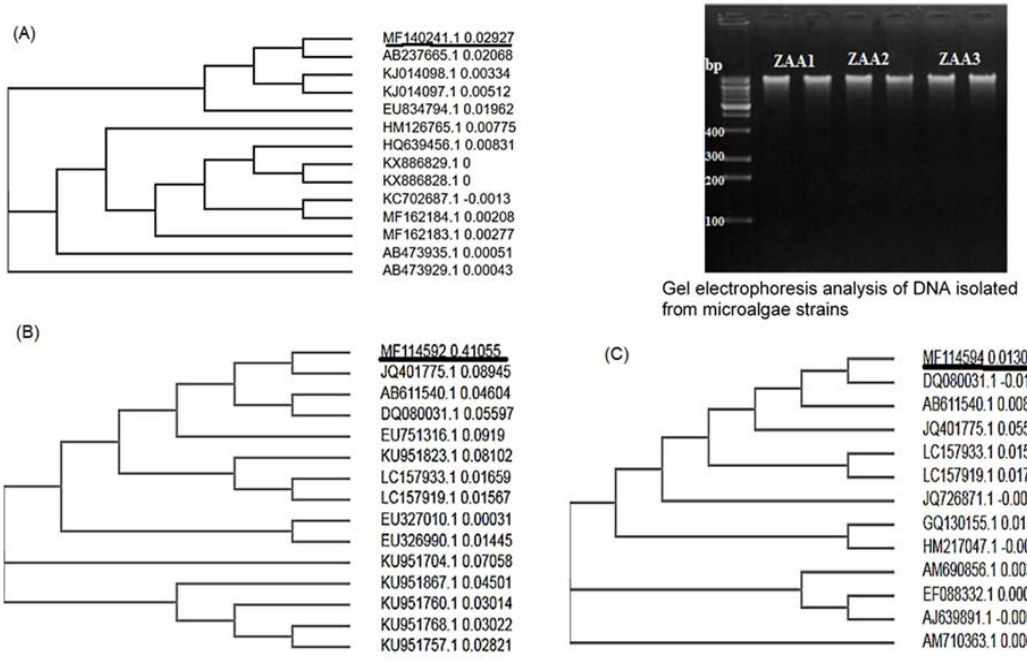

Fig. 3: Phylogenetic tree signifying the evolutionary relationship of microalgae strains. By using Clustal Omega database through neighbor joining method tree was constructed. The evolutionary relationship distances were calculated by Maximum Composite Likelihood method and are in the units of number of base substitutions per site (a) *Phormidium* sp. ZAA1 (Accession Number: MF140241) (b) *Viridiplantae* sp. ZAA2 (Accession Number: MF114592) (c) *Cosmarium* sp. ZAA3 (Accession Number: MF114594).

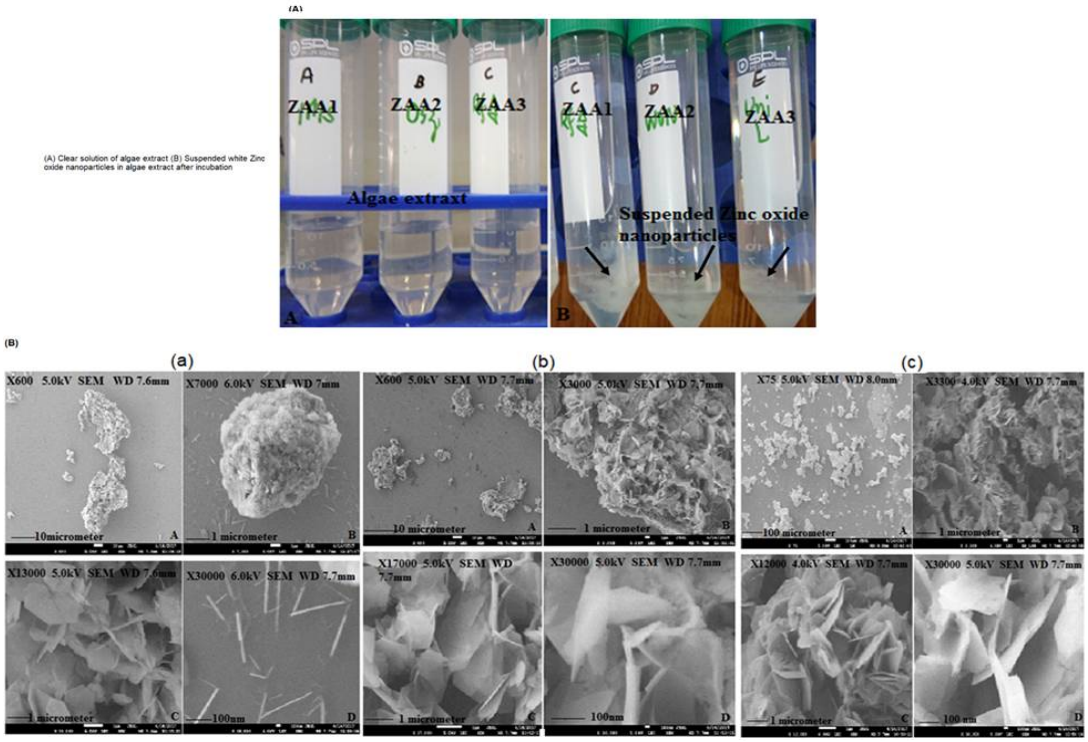

Fig. 4: (A) Synthesis of ZnO-NPs (B) SEM micrograph of zinc oxide nanoparticles synthesis by microalgae strains at different magnification (a) ZAA1 (b) ZAA2 (c) ZAA3 A. SEM image of ZnO NPs at 600X magnification B. SEM image of ZnO NPs at 7000X magnification C. SEM image of ZnO-NPs at 13000X magnification D. SEM image of ZnO NPs at 30000X magnification.

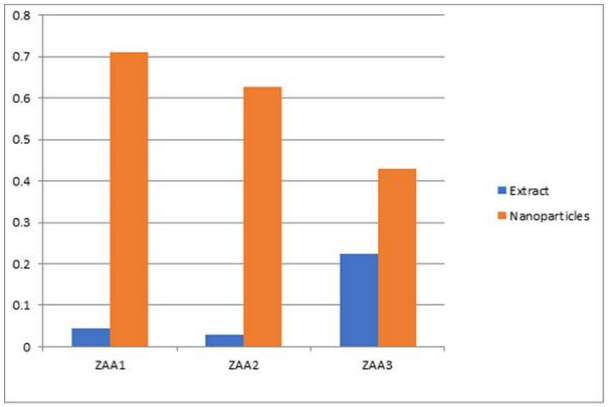

Fig. 5: Absorbance of microalgae extract and zinc oxide nanoparticle at 358nm through UV-spectrophotometer

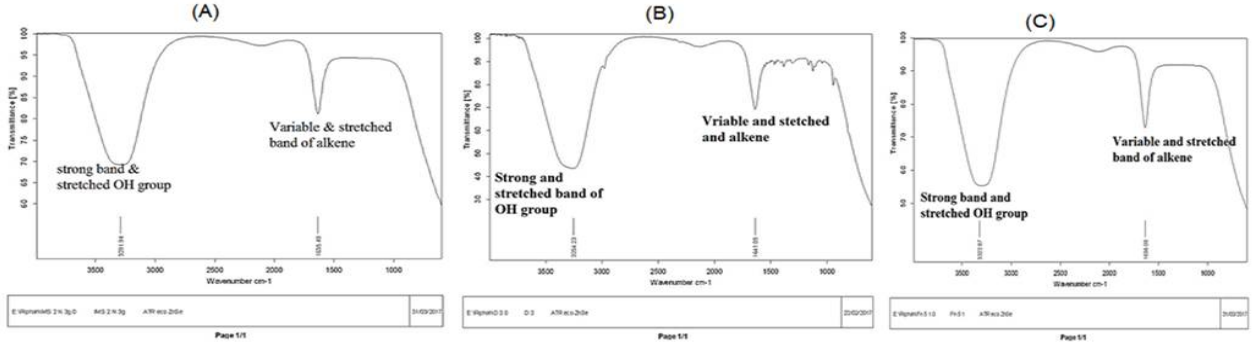

Fig. 6: FT-IR spectrum of ZnO-NPs synthesized by microalgae strains (A) ZAA1 (B) ZAA2 (C) ZAA3

Table 1: Preparation of modified BG-11 medium for culturing of microalgae strains

Stock solution 1				
No.	Chemicals	Per liter	Per 500ml	Per 250ml
1	Na_2EDTA	0.1g	0.05	0.025
2	NH_4Cl	0.6g	0.3	0.15
3	$C_6H_8O_7$	0.6g	0.3	0.15
4	$CaCl_2$	3.6g	1.8	0.9
Stock solution 2				
No.	Chemicals	Per liter	Per 500ml	Per 250ml
1	$MgSO_4$	7.5g	3.75g	1.9g
Stock solution 3				
No.	Chemicals	Per liter	Per 500ml	Per 250ml
1	K_2HPO_4	3.05g	1.525g	0.8g
Stock solution 4 (micronutrients)				
No.	Chemicals	Per liter	Per 500ml	Per 250ml
1	$MnCl_2$	1.81g	0.91g	0.45g
2	Na_2MoO_4	0.391g	0.2g	0.1g
3	$ZnSO_4$	0.222g	0.111g	0.05g
4	H_3BO_3	2.89g	1.44g	0.8g
5	$CoSO_4$	0.05g	0.025g	0.0125g
6	$CuSO_4$	0.079g	0.04g	0.02g

Table 2: Isolated microalgae strains collected from different location and conditions

S. no.	No. of Samples	Location	pH	Conditions
1.	ZAA1	From Department of IMS BZU-Multan.	7.0	Running water, attached with bottom soil
2.	ZAA2	From Aquarium (fish form department) Old Shujabad-Multan.	6.5	Light greenish stagnant water Free floating alga
3.	ZAA3	From fields of Laar-Multan.	6.0	Muddy water

Table 3: Antibacterial activity of ZnO-NPs measured in terms of zone of inhibitions (millimeter)

Name of Bacteria	ZAA1		ZAA2		ZAA3	
	NPs	Std.*	NPs	Std.	NPs	Std.
E. coli	20	40	11	40	17	41
S. aureus	14.5	18	20	23	14	17
K. pneumoniae	11	27	11	31	10	27
C. freundii	20	40	11	40	15	38
M. luteus	12	40	16	40	16	40

*Standard drug i.e. ciprofloxacin

RESULTS

Sample collection and isolation

Three strains of microalgae ZAA1, ZAA2 and ZAA3 were isolated and purified from fresh water, stagnant water and muddy water respectively. Table 2 showed the location, pH and condition of water samples from where these strains were isolated.

Characterization of isolated microalgae strains

Morphological analysis

The microalgae strains ZAA1, ZAA2 and ZAA3 were observed under microscope at 40X magnification. As a result of this analysis, strain ZAA1 appeared as thread like green filamentous indicating *Phormidium species.* The appearance of spherical clusters of coccid microalgae strain ZAA2 was ranked as *Viridiplantae species* and similarly ZAA3 was shown as round centrally distorted strain confirming *Cosmarium* species (fig. 2).

Molecular analysis

The morphologically identified strains of microalgae were confirmed at molecular level through sequence analysis of 16SrRNA region. The NCBI sequence alignment analysis ensured that isolated strains of microalgae showed 100% identity with reference genome confirming *Phormidium sp.* ZAA1 MF140241, *Viridiplantae sp.* ZAA2 MF114592 and *Cosmarium sp. ZAA3* MF114594. The phylogenetic tree was constructed by neighbor-joining method that showed evolutionary relationship with partial sequences of other microalgae strains (fig. 3).

Synthesis of ZnO-NPs

The use of ZAA1, ZAA2 and ZAA3 strains microalgae successfully produced bioactive ZNO- NPs. Filtrate color variations confirmed the production of ZnO-NPs following zinc-nitrate incubation (fig. 4A).

Antibacterial activity of ZnO-NPs

ZnO-NPs showed considerable antibacterial activities against *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Citrobacter freundii* and *Micrococcus luteus*. Nanoparticles produced from extracts of ZAA1 and ZAA3 showed considerable difference in antibacterial action as compared to ZAA2 (table 3). Ciprofloxacin was used as standard drug.

ZnO-NPs characterization

SEM analysis

SEM analysis showed the morphology and size of nanoparticles produced in 75X, 600X, 3300X, 7000X, 12000X, 13000X and 30000X from the ZAA1, ZAA2 and ZAA3 microalgae strains. fig. 4B indicated ZnO-NPs with sphere and poly-dispersive sizes ranging from 1µm up to 100nm.

UV-spectroscopy and FTIR analysis

The particles were investigated at the wavelength of 358nm. ZnO-NPs showed different absorbance values at this wavelength. The absorbance of nanoparticles was higher as compared to extract (fig. 5). FTIR was used for the study of surface chemistry and functional groups of ZnO-NPs. The absorbance band and spectra of ZnO-NPs were observed in the spectral region of 1000 to 4000cm^{-1}. In the region of 3200-3300 cm^{-1}, the alcoholic functional group (O-H), with broad intensities and a strong band, was specified. In spectral-region of 1600-1650cm^{1}, stretching robust bond of alkenes functional group (C=C) was shown (fig. 6).

DISCUSSION

Nanoparticles have a wide range of applications including electronics, pharmaceutics, metallurgy textile and others (Habeeb, 2013). Nanoparticles are synthesized using various chemical and physical methods. These processes are subject to a number of limits, such as high energy consumption, costly, incompatible, toxic byproducts and waste (Reynolds *et al.*, 2002). Because of these problems, green, organic, safe and economical techniques for the synthesis of nanoparticles with bacteria, fungi, algae and plants are developed (Sabir *et al.*, 2014). In this study, we focused to synthesize ZnO-NPs using microalgae and determined antibacterial activity of these particles.

The interesting and innovative investigation of living organisms shows that they can convert and produce simpler inorganic metal ions into complex nanoparticles. Not every biological entity can synthesize nanoparticles because it has no biological machinery to absorb metal ions in its environment. The size, form and composition of nanoparticles can be affected by cultural conditions, such like media, light, pH, temperature, and agitation. In order to manage and define nano particles production, suspicious and prudent selection of biological entities is needed (Azizi *et al.*, 2014).

Recent research has widely spread the production of nanoparticles using algae because of their synthesis efficiency, easy availability and limited research. Algae have enzyme machinery and functional groups that transform complex metallic substances by reducing them into non-toxic metallic compounds (Azam *et al.*, 2012; Shah *et al.*, 2015). Algae mainly used for the production of extracellular nanomaterial. Both fresh and marine water algal species including *Cyclotella, Euglena, Gymnodinium, Chara, Chlamydomonas Sargassum and Corallina* have the capability to produce nanoparticles (Aliya *et al.,* 2009; Rao and Gautam, 2016).

Due to diverse chemical and physical properties of nanoparticles, these important ZnO-NPs used in the pharmaceutical sector are more stable, biocompatible, less toxic, and anti-pathogenic (Nagarajan and Kuppusamy, 2013; Fawcett *et al.*, 2017). ZnO-NPs were synthesized by aqueous extract of marine brown macroalgae *Sargassum muticum,* having size ranges from 3-57nm (Yah and Simate, 2015). Temperature, pH, concentration of extract, substrate solution and incubation time are important conditions for the synthesis of controlled, clearly defined size and form of the ZnO-NPs (Emami-Karvani and Chehrazi, 2011). These nanoparticles can be characterized by UV-vis spectroscopy, FT-IR, X-Ray diffraction (XRD), transmission electron microscopy (TEM), and scanning electron microscopy (SEM). In 2013, Kuppusamy and Nagarajan reported the synthesis of ZnO-NPs using *Hypnea valentiae* (red algae), *Sargassum myriocystum* (brown algae) and *Caulerpa peltata* (green algae) (Emami-Karvani and Chehrazi, 2011). This study showed that brown algae produce ZnO -NPs efficiently in comparison with other strains. The morphological dimension of these ZnO-NPs ranged from 76-186 nm.

ZnO-NPs are effective antimicrobial agents and we determined the antibacterial activity of ZnO-NPs against *Escherichia coli*, *Staphylococcus aureus, Micrococcus luteus, Klebsiella pneumoniae, Citrobacter freundii* resulted significant zone of inhibitions (table 1). Therefore, algal synthesis of ZnO-NPs would help to treat infectious and non-infectious diseases.

ZnO-NPs were studied with a 358nm UV-Visible spectrophotometer to determine the absorption and optical properties (Manivasagan and Kim, 2015). The microscopic scan analysis reveals nanoparticles with their morphological and topological characteristics at 75, 600, 3300, 7000, 13000 and 30000X magnification. SEM micrograph sowed that it has spherical and poly-dispersive forms from 1µm to 100 nm. In order to observe functional group and surface chemistry of ZnO-NPs, FT-IR spectral analysis showed important carbonyl, hydroxyl, alkenes and alkanes functional groups (Chang and Tsai, 2008; Oscar *et al.*, 2016).

CONCLUSION

ZnO-NPs with the size of 100nm were effectively produced by easy, non-toxic and cost-effective procedure using different strains of microalgae cultures including *Phormidium sp.* ZAA1, *Viridiplantae sp.* ZAA2 and *Cosmarium sp. ZAA3*. These nanoparticles were characterized by using FTIR, UV-visible spectroscopy and SEM. These biologically active particles showed effective antibacterial activity against different strains of bacteria.

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