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Mycobiosynthesis of novel non toxic zinc oxide nanoparticles by a new soil fungus *Aspergillus terreus* VIT 2013

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ABSTRACT

Non toxic zinc oxide (ZnO) nanoparticles were synthesized using a novel soil fungus, Aspergillus terreus VIT 2013. Production of these particles in fungal filtrate was observed by a change in colour from yellow to whitish yellow, along with a white precipitate which settled to the bottom of the flasks after three days. For confirming their presence through UV spectral studies, the filtrate was scanned from 200 to 500nm and a strong peak was observed at 388nm. X-ray diffraction analysis indicated peaks at (100), (002), (102), (110) and (112) corresponding to the hexagonal structure of ZnO nanoparticles. The atomic force microscopy images showed the presence of spherically shaped ZnO nanoparticles in aggregated form. Fourier transform infrared spectrum (FTIR) revealed the presence of primary and secondary amines which probably aids in particle stabilisation. The highlight of the study was that the particles showed no antibacterial activity against normal skin flora, certain MTCC isolates and some fungal pathogens. Overall analysis of results revealed that the mycobiosynthesis of ZnO nanoparticles is a slow redox process, which may involve extracellular oxidase and reductase enzymes, synthesised by a new fungus, viz., Aspergillus terreus VIT 2013. Further, these particles are non toxic and hence can be used in the formulation of skin care products. Their applications can also be extended to other industries as disposal will not present any ecological imbalance.

Keywords: Soil fungi, *Aspergillus terreus* VIT 2013, ZnO nanoparticles, Antimicrobial activity, Skin flora

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INTRODUCTION

Nanomaterials largely exhibit distinctive physical, chemical and biological properties compared to their macro scaled counterparts [1]. Metal nanoparticles, with such unique characteristics, have aroused major interest in the field of materials science.Their synthesis and applications in fields of electronic, photonic and food industries, as well as environmental remediation, has been firmly established [2-4]. Additionally, biocompatible, functionalized and inert metal nanoparticles have found potential uses in cancer diagnosis and therapy[5]. Chemical methods have been fairly successful in synthesizing metal nanoparticles for many of these applications. However, several disadvantages are observed here which include the use of noxious precursor chemicals and toxic solvents along with the incidental synthesis of harmful by products [6]. Hence increasing efforts are being directed towards developing eco-friendly ‘‘green synthesis’’ pathways for their production [7]. These methods utilise non toxic biological organisms and clean technology, thus adopting an environment friendly approach.

Utilisation of micro organisms for the synthesis of metal nanoparticles is slowly being established, as it leads to formation of particles with well-defined shapes and sizes within a narrow size range. This process is reproducible and can be achieved successfully every time by carefully controlling and monitoring the syntheses conditions. Bacteria have been used for production of metal as well as metal oxide nanoparticles. For example, the sulfate reducing bacteria *Desulfovibriode sulfuricans* has been employed for the production of palladium nanoparticles and silver resistant bacteria has been used for synthesizing anisotropic silver nanoparticles [8,9]. Fungi are emerging as more effective and alternative organisms for this purpose as they are an established source for secretion of

extracellular enzymes which possibly reduce metal ions in the medium to nanoparticle form. Several reports attest this phenomenon [7,10,11]. However, the precise mechanism by which the particles are produced using microbes has not been reported clearly.

In this study, *Aspergillus terreus* has been utilised for the extracellular synthesis of zinc oxide nanoparticles. These particles have been characterised by various instrumental techniques and their antibacterial activity has been ascertained against skin flora and also certain common pathogens. Many metal and metal oxide nanoparticles find extensive applications in health care, pharmaceutical and cosmoceutical industries [3, 12] probably due to their antibacterial and antifungal activity. But this property may cause them to be toxic to normal skin flora too which may be deleterious to the skin in the long run as these bacteria serve to protect the skin against damage. The particles synthesised in this study are harmless and show no activity against normal skin flora. Hence they can be used safely in skin care products and to our knowledge, this is the first report for the mycobiosynthesis of skin safe ZnO nanoparticles.

EXPERIMENTAL SECTION

Chemicals and media: Zinc Chloride ($ZnCl_2$, extra pure) was purchased from Thomas Bakers and media were purchased from Hi Media.

Instruments: UV- Vis Spectrophotometer (BL222, ELICO), Powder X-ray diffraction (XRD, Brukar Advance, Germany) Fourier Transform Infrared Spectroscopy (IR Affinity, Shimatzu), Atomic Force Microscopy (Nano surf, Switzerland).

Microbial isolates: *Micrococcus luteus* (MTCC 4300), *Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 8076), *Klebsiella pneumoniae* (MTCC 7407), *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (MTCC 441), *Salmonella typhi* (MTCC 3231), *Enterobacter aerogenes* (MTCC 111), *Proteus mirabilis* (MTCC 9493), *Penicillium chrysogenum* (MTCC*160), *Aspergillus niger* MTCC 282), *Candida albicans* (MTCC*1637), *Saccharomyces cerevisiae* (MTCC 170) and *Fusarium oxysporum* (MTCC 284). skin flora and food pathogens, *viz.*, *Micrococcus* sp, *Bacillus* sp. *Penicillium* sp, *Aspergillus* sp and *E.coli* and *Salmonella* sp.

Biomass production of fungus

Aspergillus terreus VIT 2013, isolated from soil sample and characterized previously using 28S DNA sequencing (unpublished results) was used for the study. The fungi was subcultured and grown in Malt Glucose Yeast Peptone media (MGYP) with the following composition (grams/litre): Malt extract-3, glucose-10, yeast extract-3 and peptone-5. The flask was incubated for 10 days at room temperature to obtain sufficient fungal biomass. The broth was then decanted and the fungal mat was processed further by washing with Milli Q water thrice to remove the media components. The mat was reinoculated in 150ml of Milli Q water for 72 hours at room temperature. It was subsequently filtered and the filtrate was used for biosynthesis of ZnO nanoparticles.

Biosynthesis of ZnO nanoparticles and their characterisation by UV-Vis spectrophotometry

Synthesis of ZnO nanoparticles was initiated by adding 0.1mM Zinc Chloride solution ($ZnCl_2$) to 50 ml of fungal filtrate in a 100 ml conical flask. UV spectral readings were recorded for zero hour by scanning the filtrate between 200 to 800nm at one nm intervals. Subsequently, the solution was incubated in the dark at room temperature. Since production of ZnO nanoparticles was slow, scanning was continued every 24 hours. A control without the addition of $ZnCl_2$ and containing only the filtrate was maintained similarly. To check for natural substrate hydrolysis, a second control containing only 0.1mM $ZnCl_2$ was separately maintained and similarly monitored.

Instrumental analysis of ZnO nanoparticles

The filtrate containing ZnO nanoparticles was centrifuged at 10000rpm for 15 minutes. The pellet formed was washed with double distilled water and centrifuged again. The process was repeated thrice and a smear was made on a glass slide and dried. This was subjected to AFM analysis. The wet pellet was dried further at room temperature, mixed with KBr powder and scanned between 4000 to 500 cm^{-1} for functional group analysis by infrared spectroscopy. The dried powder of ZnO nanopartcles was packed to a flat surface onto a sample holder and powder XRD readings were recorded**.**

Isolation of skin flora

To check the toxicity of ZnO nanoparticles towards skin associated bacteria, skin flora was collected using sterile cotton swabs and spread on nutrient agar plates. The plates were incubated at 37^0C for 24 hours. Gram staining was carried out for the isolated colonies and their morphology was recorded. These colonies were subcultured on separates slants, biochemical tests conducted and organisms identified.

Antimicrobial activity of ZnO nanoparticles

In vitro antibacterial activity of ZnO nanoparticles was tested against skin flora and MTCC isolates mentioned above along with certain food pathogens, *viz., E.coli, Salmonella sp Micrococcus sp and Bacillus sp.* using CLSI guidelines [13]. Mueller Hinton agar plates were prepared and wells were made using sterile borer. An overnight culture of each isolate (all MTCC, skin flora and food pathogens), grown in nutrient broth was swabbed separately on different plates. ZnO nanoparticles were thoroughly washed with MilliQ water and 100µl of nanoparticles (2mg/ml) were added to each of the wells. The plates were incubated at 37^0C for 24 hours. Antifungal activity, against organisms mentioned above, was also carried out on Sabouraud Dextrose Agar (SDA) plates [14]. Wells were bored in SDA using sterile borer. Spore suspension of fungi was made in sterile water and spores were spread on the SDA plates. 100µl of 2mg/ml concentration of ZnO nanoparticles were added into each well. The plates were incubated at room temperature for seven days. The zone of inhibition was checked after the incubation time. The original fungal filtrate served as the negative control. 100µl of 0.1mM $ZnCl_2$ solution was added into the well and antibacterial activity was also measured. Plates were checked for zone of inhibition around the well.

RESULTS AND DISCUSSION

Zinc nanoparticles, with interesting and novel properties have made an enormous impact in several frontiers of science and technology [15, 16]. Being an n type semiconductor with a wide band gap (3.37 eV) and a large exciton binding energy of 60 mev at room temperature, crystals of ZnO exhibit a wurtzite (B4) structure, which is non centro-symmetric. These, along with their piezoelectric property, has proved to be very useful in the development of sensors and actuators in the opto electronic and semiconductor industries [2]. Some other versatile applications include their usage in cleaning of oil paintings [17], water remediation [4] and improving thermoelastic property and ecological friendliness of carboxylated nitrile elastomers [18]. The UV blocking feature of these particles has been successfully exploited by the textile industry for imparting finishing touches onto fabrics and by the cosmetic industry for incorporation into sunscreens and other related preparations [19, 12]. Several studies have attributed antibacterial and antifungal activities to these particles and elucidated their mechanistic modes of action [20, 14]. Also, since ZnO has been attested as a safe material by the FDA, the antimicrobial property has proved to be advantageous for its application in food packaging [3].

With a view to cater to the versatile applications of ZnO nanoparticles, in the present study, the soil fungi *Aspergillus terreus* VIT 2013 has been utilised for their synthesis. The conical flasks containing fungal filtrate, when challenged and incubated with 0.1mM $ZnCl_2$, lead to the formation of ZnO nanoparticles, as visualised from the colour change of the fungal filtrate, from pale yellow to turbid whitish yellow as shown in Fig.1.0. Neither a colour change nor precipitate formation was observed in the flask containing only either fungal filtrate or $ZnCl_2$ solution, which served as experimental controls.

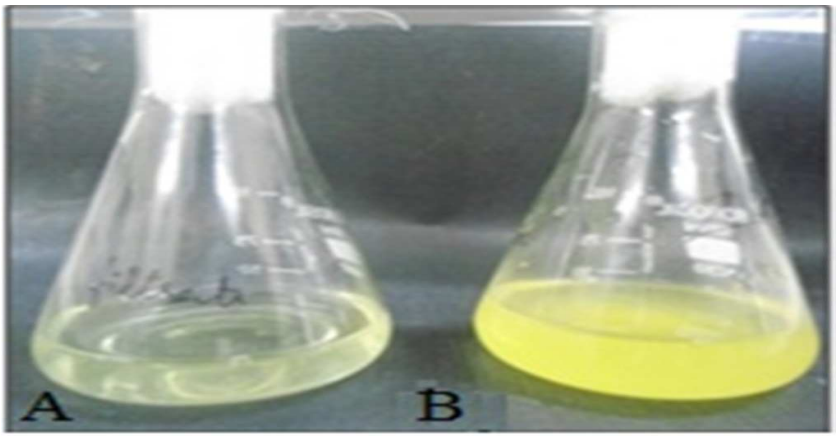

Fig. 1. Redox process of substrate to ZnO nanoparticles indicated by the colour change (A) Only filtrate (B) Filtrate and $ZnCl_2$

Synthesis in the experimental flask was observed to be a slow process, being initiated sluggishly in the system, but starting to be visible after 24 hours with weak absorption at 388 nm. Synthesis accelerates by 48 hours, as indicated by a sharp increase in absorbance at the same wavelength. The spectrum showed no other peak confirming only the formation of ZnO nanoparticles (Fig 2).

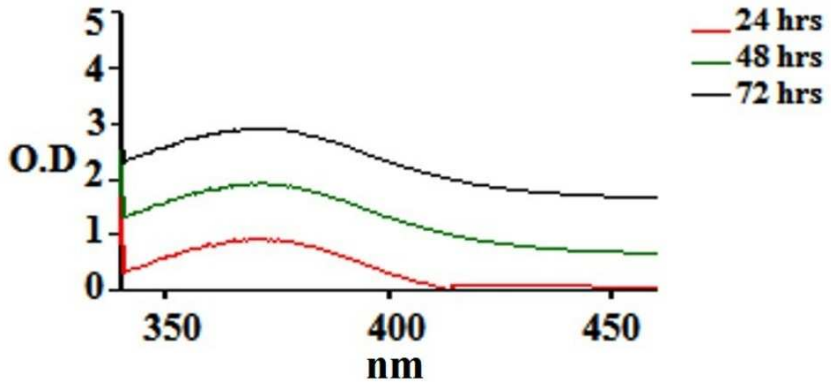

Fig. 2. UV- spectral analysis of ZnO nanoparticles scanned at 12 hours of intervals

The resultant white precipitate settled in the flask after a period of 2-3 days, probably due to aggregation and agglomeration processes. ZnO nanoparticles are known to absorb visible light between 340 and 390nm and the specific characteristic wavelength of absorption is dependent on size of the particles [21]. In case of large particles, blue shift is seen with respect to their band gaps and in our system, the particles produced seem to be nearing the upper limit of the range in which nanoscience operates. The particles are also fairly polydisperse as indicated by the broad band obtained at 388nm (Figure 2.0). This inference is also supported by the data obtained from the AFM study which puts down the size of the nanaparticles as between 28-82 nm. The AFM image is shown in figure 3.0 and the particles are seen to be spherical and aggregated in groups.

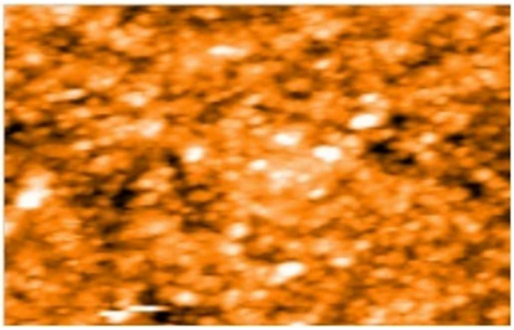

Fig. 3. AFM image of ZnO nanoparticles

It would be interesting to elucidate the mechanism by which the nanoparticles are formed in the present mycobiological system. One strong possibility highlights an important role for the electrostatic interactions, between NADH dependent reductase enzyme, secreted out amply by the fungi into the medium, and the positively charged zinc ions donated by the substrate. Such interactions have been previously reported in the literature [10,11]. A natural consequence may be the reduction zinc ions to nanoparticle form, which then subsequently combines with oxide ions from the medium to form ZnO particles, thus may be conferring greater stability to the system. Microbial reduction of metal ions to simple metal nanoparticles has been discussed by several authors [22, 23]. Previous reports from our laboratory also emphasised the synthesis of silver nanoparticles from related soil fungus, *viz*., *Aspergillus niger* [24]. It would be relevant to state here that *Aspergillus terreus* aids the synthesis of only ZnO nanoparticles and not plain Zn nanoparticles. Similar is the case with silver oxide and copper oxide nanoparticles (unpublished results from our laboratory). The reason for this phenomenon is yet to be ascertained but it strongly suggests the involvement of a second enzyme apart from the reductase enzyme, which may be present in *Aspergillus terreus* VIT 2013 and not in many other commonly used organisms reported for the synthesis of several metal nanoparticles including *Aspergillus niger* which was used in our previous study. This enzyme can probably also be an extracellular glucose monooxidase which facilitates the association of metal nanoparticles with the electronegative oxide particles in the system to form the stable ZnO nanoparticles. Energetic stability may also be conferred in the process and this may be driving force for the formation of the wurtzite structure.

Extracellular glucose monooxidase, isolated from *Aspergillus terreus*, has also been also reported [25]. The predicted mechanism for the formation of ZnO nanoparticles by *Aspergillus terreus* VIT 2013 in this study is represented in Figure 4.0.

$$O^{2-} \xrightarrow{\text{Oxidase}} O^{-} + e^{-}$$

$$O^{-} \xrightarrow{\text{Oxidase}} O^{\circ} + e^{-} \qquad \text{(Oxidation)}$$

$$Zn^{2+} + 2e^{-} \xrightarrow{\text{Reductase}} Zn^{\circ}$$

$$Zn^{\circ} + O^{\circ} \xrightarrow{\text{Reductase}} ZnO \qquad \text{(Reduction)}$$

Fig. 4. Representation of hypothetical mechanism for ZnO nanoparticle formation by *Aspergillus terreus* VIT 2013

Analysis of ZnO nanoparticles by XRD revealed the *2θ* peak values at 30^{O}(100), 34^{O} (002), 47^{O}(102), 55^{O}(110) and 75^{O}(112). This can be assigned to the hexagonal ZnO wurtzite structure as supported by the standard data of JCPDS card no. 36-1451[26]. The peaks of XRD strongly supports the biofabrication ZnO nanoparticles even though the intensity and heights of the peaks are found to be lower in comparison with reported values (Fig.5).

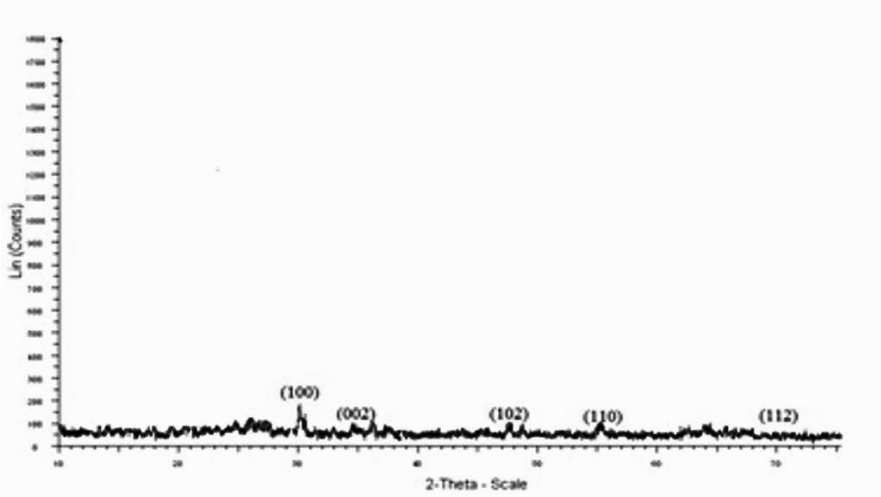

Fig. 5. XRD peaks of ZnO nanoparticles (JCPDS card no. 36-1451)

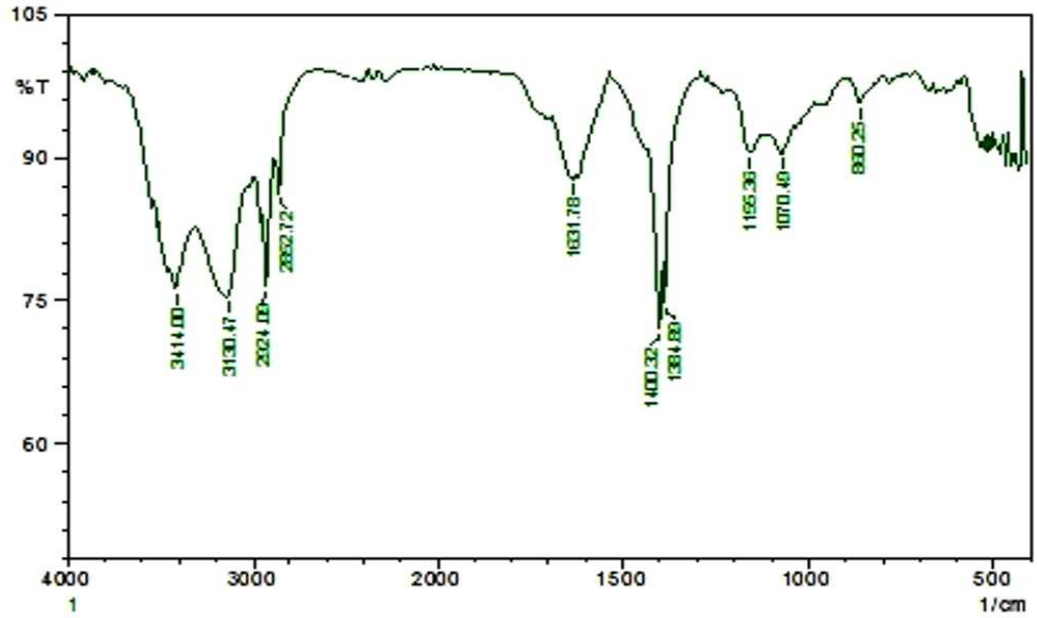

Fig. 6 Determination of functional groups involved in the ZnO nanoparticles by FTIR spectroscopy

It has been reported that biosynthesis of metal or metal oxide nanoparticles are mediated through microbial enzymes**.** In the current study, involvement of enzymes was verified through FTIR peaks obtained at 3414 cm^{-1} and 3130 cm^{-1} which correspond to primary and secondary amines respectively. The peaks at 2924 cm^{-1}, contribute towards C-H bending of aromatic ring. The peak at 1631 cm^{-1} corresponds to C=O group of amide I and the peaks of amide II and amide III were found at 1402 cm^{-1} and 1135 cm^{-1} respectively [27]. The presence of Zn-O interaction was confirmed by the peaks at 1070 cm^{-1} and 860 cm^{-1} which are near to peak 979.9 cm^{-1} (Fig 6) [28]. Association of the above functional groups with nanoparticles strongly reveal the involvement of proteins in the formation of ZnO nanoparticles. These functional groups may be present in the extracellular oxidase and reductase enzymes which play an important role in catalysing the reaction for the formation of ZnO nanopartcles. Involvement of reductase enzyme in the synthesis of metal nanoparticles is well known. Based on our results we propose the new hypothesis of involvement of both oxidase and reductase enzymes in the biosynthesis of ZnO nanoparticles.

Though there are several reports predicting the antimicrobial activity of ZnO nanoparticles [29], no activity was observed by us, against several MTCC isolates and skin flora, in present study. But in our previous study of the biosynthesis of Ag_2O nanoparticles by same species, good antibacterial activity against methicillin resistant *Staphylococcus aureus* was observed (unpublished results) as shown in Fig 7. On comparing the biosynthesis procedures of Ag_2O and ZnO nanoparticles by *Aspergillus terreus* VIT 2013, it has been noticed that, the biosynthesis of the former particles is a rapid process and they are produced within an hour. In case of ZnO nanoparticles, more than three days are required for their production. Even though in both cases, the redox mechanism is expected to operate, as only the metal oxides are preferred to be formed instead of metal nanoparticles, the slow rate of synthesis of ZnO by the enzymes may have rendered them non toxic. This makes them more advantageous for use in cosmetics as they do not harm the resident skin flora.

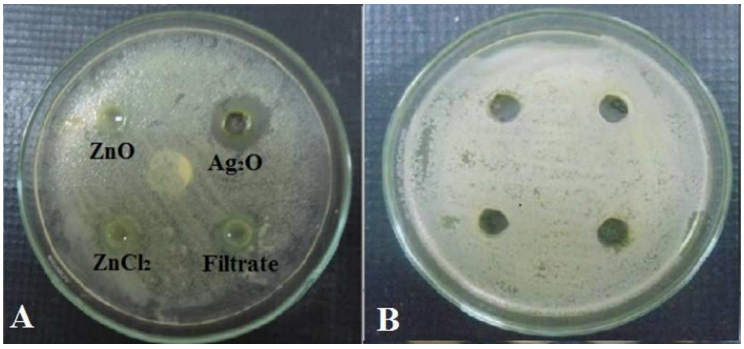

Fig. 7. Antimicrobial activity of ZnO Nanoparticles, $ZnCl_2$ (control I) and fungal filtrate (control II) against A. MRSA B. MTCC culture of *S. aureus* ((MTCC 3160) isolate

The non toxic nature of these ZnO particles were confirmed by assessing their activity against certain fungal food pathogens, *viz.*, *Micrococcus* sp, *Bacillus* sp. *Penicillium* sp, *Aspergillus* sp and *E.coli* and *Salmonella* sp. Here too the particles showed no toxicity.

CONCLUSION

ZnO nanoparticles are reported to be active against many pathogenic bacteria and fungi. In addition, they may also be toxic to the skin flora. Despite their toxicity, they are used in many formulations in cosmetic and heath care industries. Further, these particles also have other widespread applications in many areas, and their disposal after use, may cause an ecological imbalance and health hazards. The zero toxic and soft ZnO nanoparticles, synthesised in present study, by a new soil fungus *Aspergillus terreus* VIT 2013 can be used safely by the cosmetic and pharma industries. Also, disposal of these particles, by various other industries, after the relevant application, is not expected to damage the ecosystem or cause any deleterious effects in humans.

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