Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN 0976-9595

Research Article

BIOSYNTHESIS OF ZINC OXIDE NANOPARTICLES BY ENDOPHYTIC FUNGI *ASPERGILLUS NIGER* AND THEIR POTENTIAL ANTIBACTERIAL EFFECTS ON *PROPIONIBACTERIUM ACNES*

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ABSTRACT

Acne is a worldwide chronic inflammatory skin condition caused by *Propionibacterium acnes* (*P. acnes*) majorly affecting teenagers and adults worldwide. *P. acnes* is a Gram positive, non-motile, non-sporulating and micro-aerophilic bacillus normally present on human skin. Elevated production of sebum by hyperactive sebaceous glands or blockage of the follicle can cause *P. acnes* to grow and multiply causing pimple or acne. The side effects of chemical substances in topical ointments and growing resistance of microorganisms to antibiotics has renewed a great interest in investigating bactericidal properties of potent nanoparticles and their nanocomposites as an alternative treatment method. Zinc has always been preferred to treat skin ailments in its various forms. Topical creams made up of Zinc sulfates and oxides have been used to treat acne but skin irritation posed a major setback. Since permeation increases at "nano" levels, Zinc Oxide nanoparticles (ZnONPs) have been used in sunscreens for their ability to block UV rays without leaving white residue when applied on the skin. The present study evaluated the efficacy of the antimicrobial activity of ZnONPs against acne-causing bacterium *P. acnes*. ZnONPs were produced by green synthesis utilizing the endophytic fungi, *Aspergillus niger*. Produced ZnONPs were characterized by UV-Vis Spectrophotometer, FTIR, EDAX and SEM for confirmation. The antibacterial effect against *P. acnes* was significant and if incorporated in sunscreens and other skin care products, ZnONPs can be a boon to cosmetic industry.

Keywords: *Propionibacterium acnes,* ZnONPs, FTIR, Antimicrobial activity.

1. INTRODUCTION

Acne affects the sebaceous follicles in the face, chest, shoulders and back. The causative agent behind this skin inflammation is a bacterium called *P. acnes* which is a human skin commensal that prefers anaerobic growth conditions and is involved in the pathogenesis of acne. Conventionally it is treated with either creams or gels having large number of side effects on patients [1]. Four factors are generally believed to be the contributors to the development of acne: increased sebum production, comedo formation in which the follicular infundibulum hyper cornifies, hyper keratinizes, and hypo desquamates. Colonization of the follicle by anaerobic *P. acnes* with its host inflammatory response was studied earlier [2]. The *Propionibacterium* has high lipolytic activity and liberates free fatty acids from sebum lipids which cause the inflammation. The most widely used treatment of acne includes benzoyl peroxide, antibiotics, anti-seborrheic medications, sulfur, keratolytic soaps and nicotinamide [3]. These treatments exhibited side effects such as skin sensitivity reactions, skin burning, scarring, bleaching, etc. As acne often requires long-term treatment with antibiotics, there are concerns that the development of resistance by *P. acnes* may be associated with conventional treatment [4]. Hence, always there is a constant thrust to find suitable antimicrobial alternatives to overcome these shortcomings.

Zinc has been used both topically and systemically to treat acne ever since history recognized its antimicrobial effects but a major disadvantage it posed was local skin irritation and nausea, when administered orally. Nano-technology has made it possible to use ZnO in a nano level which not only increases the effectiveness but also nullifies the risk of having potential side effects like skin irritation. ZnONPs have become one of the most popular metal oxide nanoparticles in biological applications due to their excellent biocompatibility, economic, and low toxicity [5]. Because of the strong UV absorption properties of ZnO, they are increasingly used in personal care products, such as cosmetics and sunscreen lotions

[6]. ZnONPs approval by the Food and Drug Administration (FDA, USA) vouch for its biocompatible nature [7]. ZnONPs have superior antibacterial, antimicrobial, and excellent UV-blocking properties; hence are much preferred in cosmetic industries. A key unresolved issue with ZnONPs is their local safety, which suggests that they may penetrate the stratum corneum barrier, gain access to viable epidermis cells, and cause potentially toxic responses. However, studies showed that repeated application of ZnONPs to human skin *in vivo* over several days did not result in nanoparticles penetration through the stratum corneum or cause any visible morphological or redox changes. This finding extended the observations that single ZnONPs applications to human skin *in vivo* are associated with minimal skin penetration and local toxicity [8]. To exploit applications of ZnONPs for treating acne, the present study evaluated the production of ZnONPS using endophytic fungi *A. niger*, thus produced nanoparticles were characterized and assessed for the antimicrobial activity against *P. acnes* that causes acne.

2. MATERIAL AND METHODS

2.1. Fungi used and growth conditions

Bark pieces of Mango tree were collected from Garden City University, Bangalore, Karnataka, India. The bark pieces were gently washed several times with tap water to remove adherent dirt and then cut into small pieces. Surface sterilized by immersing into 70% ethanol for 30 sec, followed by 0.01% mercuric chloride ($HgCl_2$) for 5 min and then soaked in 0.5% sodium hypochlorite solution for 2-3 mins, then finally rinsed with sterile distilled water and blot dried with sterile filter paper, the cut pieces of the bark were placed on Petri dish containing Potato Dextrose Agar (PDA) with streptomycin sulfate (250mg/mL) to suppress the bacterial growth and incubated at 28°C for 6-8 days for the growth of endophytes. Fungi which grew out of the bark was isolated and pure cultured onto PDA plates and the fungi was identified based on its morphological and reproductive characters using standard identification manual.

2.2. Production of ZnONPs using endophytic fungi

Fungal isolates were inoculated into MGYP (Malt extract, Glucose Yeast extract, Peptone) broth and incubated at 25°C for 2 weeks. After 2 weeks, the fungal mat was separated from broth and washed with sterile distilled water 5 to 6 times followed by the addition of 100 mL of sterile distilled water and incubation for 3-4 days. After 3 days of incubation, the fungal mat was discarded and the filtrate was collected. 100 mL of 1.5mM $ZnSO_4$ salt was added to 100 mL of filtrate [9]. White flake like precipitate was deposited at the bottom of the flask after 48 hours of incubation. White aggregate formed was filtered and centrifuged at 8,000 rpm for 10 min.

2.3. Characterization of synthesized ZnONPS

The ZnONPs synthesized from were characterized by visual observation, UV-VisSpectroscopy, FTIR, SEM and EDAX.

2.4. Antibacterial activity of ZnONPS

For antimicrobial studies, *P. acnes* culture (MCMB-855) was procured from Agharkar Research Institute, Pune, Maharashtra, India. The culture of *P. acnes* was grown in Blood Heart Infusion broth and incubated at 37°C for 24 hours. The microbial culture was swabbed onto solidified nutrient agar media for the preparation of the lawn. Wells were made using a sterile gel puncture. Different concentrations 20 µL, 40 µL, 60 µL, and 80 µL of ZnONPs were added into each corresponding well using a sterilized micropipette. The standard antibiotic erythromycin and Zinc oxide nanoparticles were prepared in the concentrations of 1mg/mL. The plates were incubated at 37°C for 24h. The antimicrobial activity was assessed by the zone of inhibition around the sample loaded in the wells.

3. RESULTS AND DISCUSSION

3.1. Production of ZnONPs using endophytic fungi

The fungal endophytes grown on PDA media were subcultured and maintained on agar slants. Out of ten fungal isolates (Fig.1) four isolates showed the production of ZnONPs. One fungal isolate was selected based on its rapid synthesis of ZnONPs with maximum absorption spectra and microscopically identified as *A. niger* (Fig. 2). The selected fungus was grown for two weeks in MGYP broth and incubated for 48 to 72 h. After 2 weeks, the fungal mat obtained was filtered and washed with sterile distilled water for 5 to 6 times followed by the addition of 100 mL of sterile distilled water and incubation for 3-4 days till it gets free from broth components. After three days of incubation, 100 mL of the fungal extract was collected and 100 mL of 1.5mM $ZnSO_4$ salt was added to it and incubated. White flake like precipitate was deposited at the bottom of the flask within 48 to 72 hours of incubation (Fig. 3). White aggregate formed was filtrate and centrifugation at 8,000

rpm for 10 min and was used for further characterization studies (Fig. 4).

Fig. 1: Endophytic Fungi

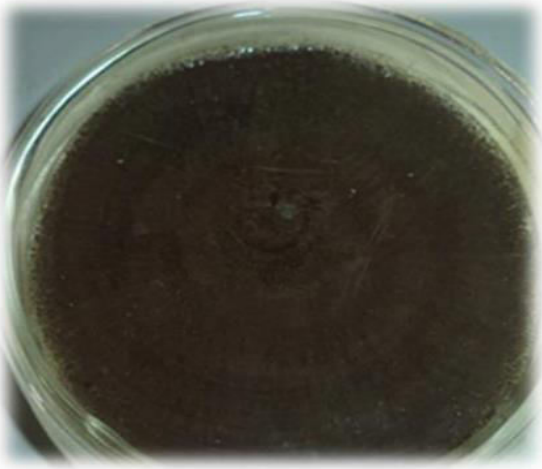

Fig. 2: ***Aspergillusniger***

Fig. 3: White aggregate of ZnONPs

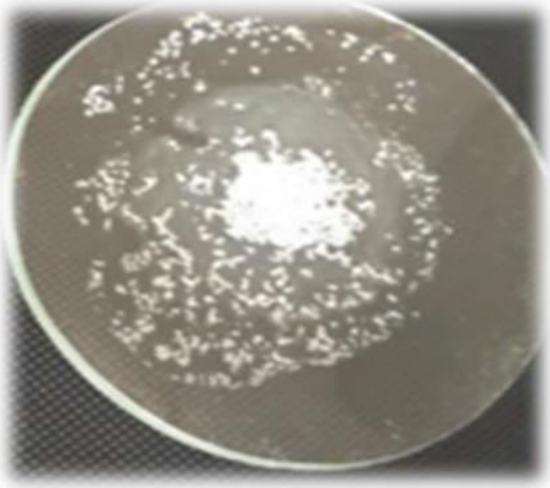

Fig. 4: ZnONPs after centrifugation

3.2. Characterization of synthesized ZnONPs

3.2.1. UV-Vis spectrum of zinc oxide nanoparticles

The synthesis and stabilization of ZnONPs produced from isolated fungus *A. Niger* was studied using UV-absorption spectrophotometer where a strong peak at about 231 nm was observed.

Zinc nanoparticles normally show a broad peak in the UV-Visible spectrum in the range of 230-330 nm [10]. Harish et al Synthesized ZnONPs from Rhizosphere soil and characterized with UV-Spectrophotometer. The absorp-tion maximum in the study was found to be 260 nm [11]. Mohd AR [12] Analyzed the optical properties of ZnONPs by UV-Spectrophotometer which showed a characteristic peak at 372 nm wavelength.

3.2.2. FT-IR spectrum analysis of ZnONPs

FTIR measurement was carried out to identify the potential biomolecule in enzyme filtrate responsible for the ZnONPs production. The peaks indicated the characteristics functional group present in the synthesized ZnONPs. Fig. 5 shows characteristic peaks at 3453. 61cm^{-1}, 2073.35cm^{-1}, 1641.93cm^{-1} and 566. 65cm^{-1}. The absorption peak at 566.65cm^{-1}corresponds to C-Cl stretch. The peak at 1641.93cm^{-1} is described to the stretching vibration of N-H bond of primary amine, alkyl C=C stretch, open chain amino group. The peak at 2073.35cm^{-1} indicates carbonyl-N=C=S stretch. The peak at 3453.61cm^{-1} is ascribed to the stretching

vibration of OH bond of alcohols, phenols, aromatic primary amines.

The presence of these functional groups makes synthesized ZnONPs as effective antimicrobial agent [13]. The study of Manjunath *et al.,* 2013 suggested the biosynthesis of metal or metal oxide nanoparticles are mediated through microbial enzymes and their studied showed involvement of enzymes in ZnONPs production which were verified through FTIR and peaks were 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. While Wang *et al.,* 2009 observed the peak at 1631 cm^{-1} which corresponds to C=O group of amides I and the peaks ofamide II and amide III were found at 1402 cm^{-1} and 1135 cm^{-1} respectively [15]. Hong *et al.,* 2006 reported Zn-O interaction showing the peaks at 1070 cm^{-1} and 860 cm^{-1} which are near to peak 979.9 cm^{-1} [16].

3.2.3. EDAX analysis of ZnONPs

EDAX provides information about the chemical composition of the sample. EDAX spectrum of ZnONPs showed 8 peaks which are identified as zinc, oxygen, calcium, carbon, iron, silicon, chlorine and phosphorus. The percentage of zinc and oxygen being the highest, 38.7 and 28.98 respectively proves the presence of impurities (Fig. 6).

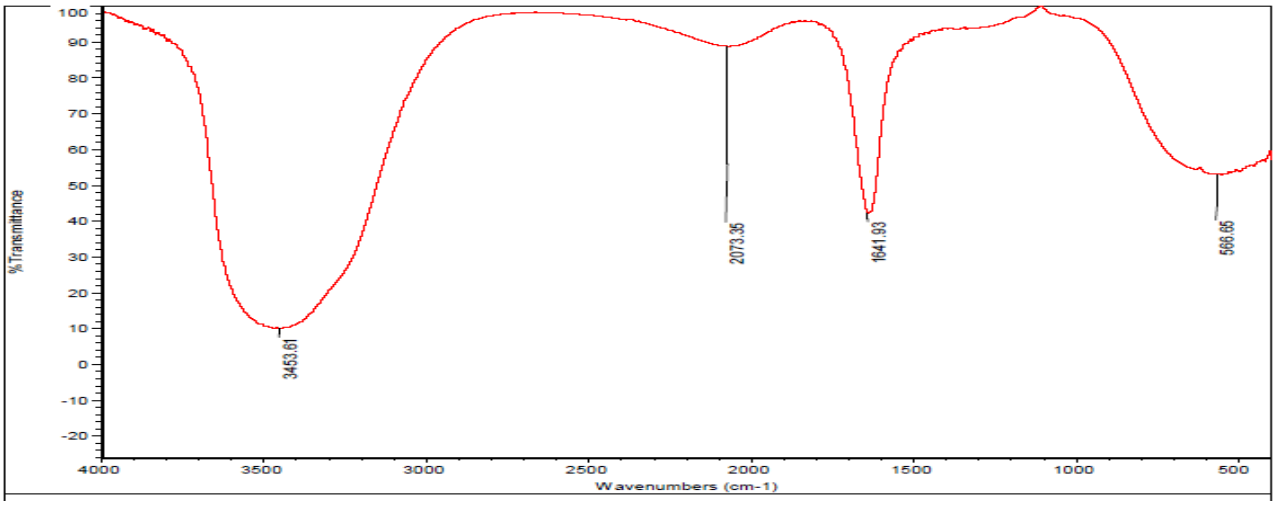

Fig 5: FTIR spectra of synthesized ZnONPs

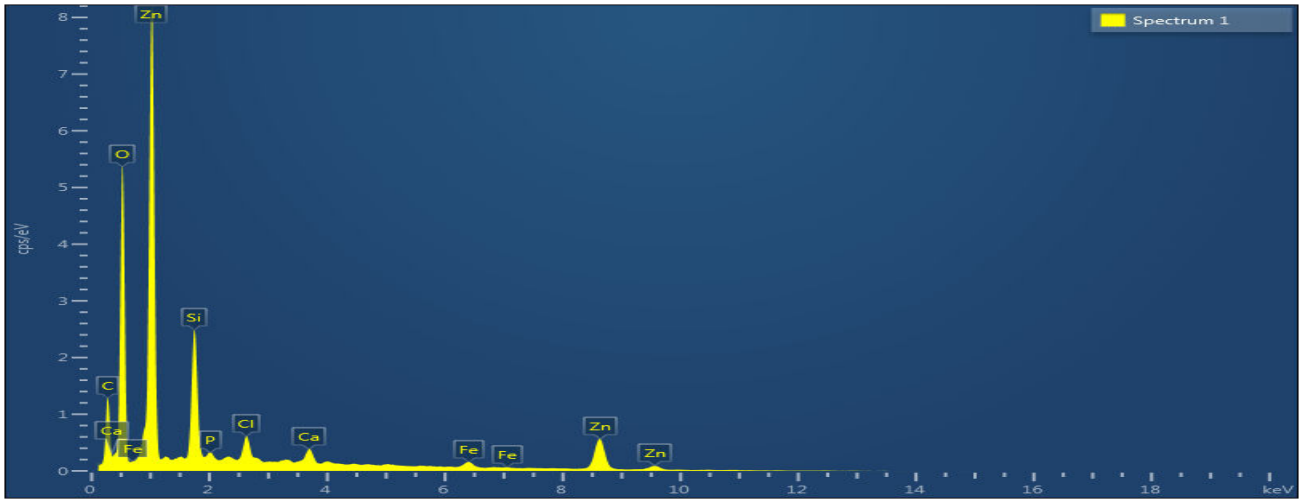

Fig 6: Elemental analysis of ZnONPs using EDAX

Aisha et al. synthesized ZnONPs using *A. Niger* and their EDX study showed the presence of elements Zn and O. The Zn content was 85.9% while O content was 8.6% and indicated that ZnONPs were pure with only traces of impurities [17].

3.2.4. SEM analysis of ZnONPS

Structural characterization of ZnONPs was analyzed using SEM. The SEM images of ZnONPs by *A. niger*is shown in Fig 7. It can be inferred that the ZnONPs are spherical shaped with an average size of 40-65 nm. Rajan et al., 2016 observed that synthesized ZnONPs using *Aspergillus fumigatus* JCF and characterized nano-particles using SEM. The ZnONPs in their study showed an average size of 60-80nm [18]. Aisha et al., reported production of ZnONPs using *A. niger* and the size of nanoparticles was found to be 66 nm with uniformly sized hexagonal morphology [19]. ZnONPs exhibit tendency to agglomerate in aqueous solution and develop soft agglomerates but this agglomeration does not create complexity as the application purposes of ZnO depend on particle size and not on agglomerate size [20].

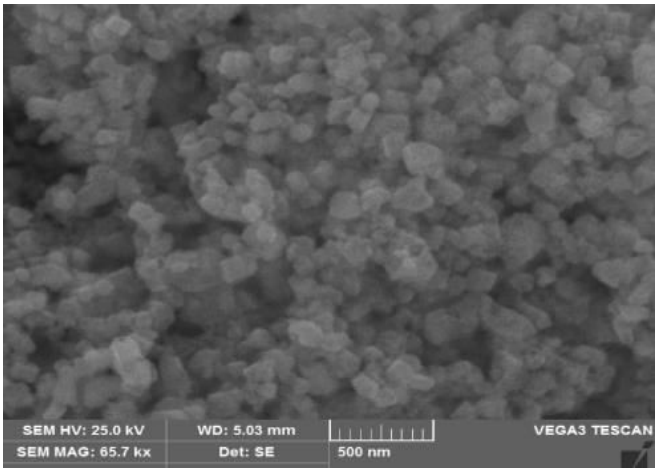

Fig 7: SEM image of ZnoNPS

3.3. Antibacterial activity of ZnONPs

The effect of ZnONPs on *P. acnes* was studied using well diffusion method. Different concentrations of ZnONPs were prepared and assessed for antimicrobial effects on *P. acnes*. ZnONPs have shown significant effects on P. *acnes*. At the concentration of 80µL, the zone of inhibition was found to be 19mm (Fig.8) whereas with the standard antibiotic the zone of inhibition was 19mm (Table 1). With increase in the volume of ZnONPs, it was observed that the zone of inhibition also increased.

Fig 8: Antimicrobial effects of ZnONPs on *P. acnes*

Table 1: Effect of ZnONPs on *P. acnes*

Concentration of ZnONPs (in µL)	Zone of Inhibition in mm
20 µL	09mm
40 µL	12mm
60 µL	14mm
80 µL	16mm

Studies conducted by researchers suggested that the main antibacterial toxicity mechanisms of ZnONPs could be possibly based on the ability to induce excess ROS generation such as superoxide anion, hydroxyl radicals and hydrogen peroxide production [21, 22]. Study of Shailaja et al reported that ZnONPs synthesis by chemical method and assessed antibacterial effects of ZnONPs on *P. acnes* have great potential of becoming safe and effective for topical application in treating acne vulgaris and other *P. acnes* associated diseases. Ohira and Yamamoto found antibacterial (*E. coli* and *S. aureus*) activity of ZnONPs on *E.coli* and *S.aureus* and revealed that the small crystallite sizes nanoparticles were stronger than those with large crystallite sizes [23].

4. CONCLUSION

Present study demonstrated the synthesis of ZnONPs using an endophyte *A. niger*. ZnONPs was synthesized utilizing *A. niger,* confirmed the absorption peak at 231 nm. SEM analysis showed spherical structure of ZnONPs of size ranging from 60-80 nm. The functional group present in the synthesized ZnONPs was confirmed by FT-IR. Antimicrobial studies revealed that the synthesized ZnONPs are effective against pathogenic *P. acnes*. ZnONPs are one of the most important metal oxide nanoparticles popularly employed in various fields due to their peculiar physical and chemical properties.

The present study highlighted the futuristic and novel way of treating acne in an effective way without the use of any antibiotic. Hence, this study concludes that *A. Niger* mediated ZnONPs may be used as effective control tool against acne causing *P. Acnes* which suggest-sits potential application in the cosmetic and skin care industry.

Conflict of Interest

Authors declare there are no conflicts of interest.

5. ACKNOWLEDGEMENT

Authors are thankful to the Vice Chancellor of Garden City University, Bangalore, for providing the necessary laboratory facilities to carry out the work.

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