4. **Biocidal mode of action study of ZNPs**

4.1. Antifungal mode of action:

4.1.1. Introduction:

Recent advances in the field of nanotechnology, particularly the ability to prepare highly ordered nanoparticulates of any size and shape, have led to the development of new biocidal agents. Nanoparticle metal oxides represent a new class of important materials that are increasingly being developed for use in research and health-related applications. Highly ionic metal oxides are interesting not only for their wide variety of physical and chemical properties but also for their biocidal activity. Several studies have indicated that metal oxides nanoparticles can be used as effective biocidal materials [1, 2, 3, 4, 5, and 6]. Like other metal oxide nanoparticles Zinc oxide nanoparticles (ZNPs) are also used as biocidal agents but a few studies have been carried out related to antifungal mode of action of ZNPs. In this chapter, detailed mode of action of newly synthesized Zinc oxide nanoparticles [ZNP (T)] has been shown. Fungitoxicity of ZNPs is mainly related to their nanosize that causes membrane disorganisation, generation of reactive oxygen species (ROS) and oxidative damage of protein.

4.1.2. Experimental:

(a) Determination of ROS: superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase activity assay:

Super oxide dismutase activity was measured by its ability to inhibit photochemical reduction of NBT at 560 nm following the method of Beauchamp *et al.*, 1971 [7]. One enzyme unit of SOD was defined as that amount of protein (in mg) causing a 50% inhibition of photoreduction. Catalase activity was estimated according to Bergmeyer, 1970[8] which checked out the initial rate of disappearance of \( \text{H}_2\text{O}_2 \) at 240 nm. Ascorbate peroxidase activity was measured following Nakano *et al.*, 1981 [9] which depended on the decrease in absorbance at 290 nm as ascorbate was oxidized. Glutathione reductase enzyme activity was evaluated spectrophotometrically at 25°C[10]. One enzyme unit of glutathione reductase was defined as the oxidation of 1 mmol NADPH per min under the assay condition.
(b) Total thiol (–SH) content assay:

Total thiol content was determined by a modified method of Ellman, 1959[11]. Briefly 0.5 g of fungal mycelia (grown at different ZNPs concentrations along with control) for each sample was taken and ground using a cold mortar and pestle. Then 4mL of 50mM ice-cold phosphate buffer (pH 7.0) was added to each sample into the mortar and mixed well. The homogenate was centrifuged at 2000g for 20min at 4°C. 3 mL of the supernatant was mixed with 2 mL of phosphate buffer (pH7.0) and 5mL distilled water and they were mixed well to get 10 mL reaction mixture. 20 μL of 0.01M DTNB solution was added to 3mL of the reaction mixture, shaken well and absorbance was recorded at 412 nm. Thiol content was calculated using extinction coefficient 13, 600 M⁻¹ cm⁻¹ for DTNB at 412 nm.

(c) Lipid peroxidation assay:

Malondialdehyde (MDA) content, final product of lipid peroxidation was measured following the slightly modified method of Dhindsa et al., 1981 [12]. Fungal extracts were prepared following the same method of extraction for thiol content assay. Then 0.5 mL supernatant of fungal extract was added to 1 mL of 20% (v/v) trichloroaceticacid and 0.5% (v/v) thiobarbituric acid. The mixture was heated in a water bath at 95°C for 30 min followed by cooling at room temperature. After centrifugation at 10, 000 g for 10min, the supernatant was used for absorbance measurements at 532 and 600 nm respectively. The absorbance for non specific absorption at 600nm was subtracted from the value at 532 nm. The amount of MDA was calculated using the adjusted absorbance and the extinction coefficient 155 mM⁻¹ cm⁻¹ [13].

(d) Proline assay:

Proline assay was evaluated following the modified method of Chinard, 1952 [14]. Control and ZNPs treated fresh mycelia were ground with an ice-cold mortar and pestle and extracted with 4 mL of 3 % sulfosalicylic acid. After homogenization and centrifugation at 2000 g for 20 min, 2 mL of the supernatants were pipetted out into a10 mL screw capped glass test tube. 2 mL glacial acetic acid and 2mL acid-ninhydrin reagent were added to the supernatants. After heating to 100°C for 45 min these screw capped tubes were placed in an ice bath to cool to room temperature. Then 4 mL toluene was added to each tube and shaken vigorously for 2 min. Then samples were allowed to rest for 15 min.
for complete phase separation. Upper toluene layer was separated and kept at room temperature for 10 min and the red colour intensity was read at 520 nm against toluene blank. The proline concentration was determined from a standard curve and calculated on a fresh weight basis (mM proline/gFW).

(e) Measurement of protein oxidation by DNPH assay:
Protein carbonyl content was a marker of oxidative stress which was measured spectrophotometrically following slightly modified DNPH binding assay [15]. All fungal strains were cultured in control and ZNPs treated PDB; then incubated at 28°C-30°C with vigorous shaking for 48 h to form mycelial balls. After 48 h of incubation mycellial balls were isolated from culture and washed with 50 mM phosphate buffer (pH 7.0). The sample balls were then freeze dried with liquid nitrogen and ground with an ice-cold mortar pestle using 8 mL of 50 mM phosphate buffer (pH 7.0). The homogenates were centrifuged at 5000 rpm for 20 minutes at 4°C. The supernatants were incubated with DNPH for 1 h at 37°C; proteins were precipitated in 10% cold TCA and washed with ethanol: ethyl acetate (1:1) to remove excess DNPH. Finally samples were dissolved in 6 (M) guanidine chloride (pH 2). The optical density was measured at 380 nm and the carbonyl content was calculated using a molar extinction coefficient of 21 mM⁻¹ cm⁻¹, resulting in final measurement in nanomoles of DNPH incorporated (protein carbonyls) per mg of protein.

(f) Fungal protein content study by FTIR spectra:
For FTIR analysis fungal strain (A. niger, MTCC -10180) was cultured as described in DNPH binding assay up to washing of mycellial ball with phosphate buffer (pH 6.8-7.0). Then mycelial balls were washed successively with deionized water and 40% ethanol. After that all the samples (both control and treated) were sonicated for 30 minutes to rupture the fungal cell wall. Finally FTIR data were taken from the liquid samples (2-3 μL) after successful air drying[16].

(g) Microarray analysis of ZNPs treated A. niger:
Normalization (Percentile shift) was done using GeneSpring GX Version 11.5 using recommended Per Chip and Per Gene Normalization. Percentile shift normalization is a global normalization, where the locations of all the spot intensities in an array are adjusted. This normalization took each column in an experiment independently, and computed the percentile of the expression values for this array,
across all spots (where n has a range from 0-100 and n=50 is the median). It subtracted this value from the expression value of each entity.

**(h) Detection of Protein Carbonylation by Westernblotting:**

Control and treated fungal samples were ground in a chilled mortar (0.5 g fresh weight ml⁻¹) in protein extraction buffer (PEB) containing 100 mM Tris-HCL, pH 8.0, 2 %%(v/v) 2 mercaptoethanol, 5 mM EGTA, 1 mM EDTA, 1 mM AEBSF, 10 g ml⁻¹ leupeptin, 1 mM p-amino-benzamidine, 5 mM 6-aminohexanoic acid, 2 μM E64, 10 μM NaF, 1 mM DPTA and 1 mM BHT. Fungal soluble protein extracts were mixed with two volumes of 10 mM DNPH in 2 M HCl at room temperature for 30 minutes with gentle agitation. A control sample was mixed with two volumes of 2M HCl, five volumes of ice-cold phenol (Tris-buffered, pH 7.9) was added to each tube respectively. After vortexing for 1 min, the mixture was centrifuged for 10 min at 10,000g. The upper phase was removed and discarded leaving the interface intact and the phenol phase was re extracted twice with ice-cold Tris-HCl buffer (50 mM, pH 8.0). Five volumes of cold 0.1 M ammonium acetate in methanol was added to the lower phase and incubated at -20°C overnight. In the following day, resulting pellets were washed three times with 1 ml 0.1 M ammonium acetate in methanol and once with 1 ml cold ethanol. To each pellet, IEF buffer [containing 7 M urea, 2 M thiourea, 4% v/v CHAPS, 65 mM DTT, 20 μl 0.1% bromophenol and 20μl IPG buffer, pH 4-7] was added. The samples were incubated for 2 h at room temperature. Insoluble material was removed by centrifugation at 10000 g for 20 min at 4°C and the supernatant was transferred in to a fresh tube for gel electrophoresis[17]. SDS-PAGE was performed with 12% gels with a loading of 10μg protein samples in each lane. Resolved proteins were electrophoretically transferred to Immobilon-P (PVDF, Millipore) membranes and oxidized proteins were detected by using anti DNP antibodies [Oxiblot Protein detection kit (Millipore)].

**(i) Zn²⁺ release from ZNPs:**

Zn²⁺ release from ZNPs was monitored at the pH of PDA medium at different time interval using inductively coupled plasma mass spectroscopy (ICPMS) as described by Xia et al. [18]. ZNPs were
mixed with 25 mL aqueous medium of pH 5.5 - 6 and allowed to stir for 24, 48 and 72 h respectively. 1.5 mL of the above aliquots was centrifuged at 25,000 rpm at 4°C after 24, 48 and 72 h of stirring and 0.1 mL of these supernatants were mixed with 0.9 mL of water. These solutions were digested with plasma pure HNO₃ and subjected to ICPMS analysis using a standard as described by Xia et al. [18].

(j) Bioavailability/uptake study of ZNPs:

(j) 1. SEM, HR-TEM and Confocal microscopic analysis:
For Scanning Electron Microscopic (SEM) and High Resolution Transmission Electron Microscopic (HR-TEM) the fungal samples (A. niger, MTCC-10180) were cultured in PDA medium and after 48 h of inoculation, fungal cultures (A. niger, MTCC-10180) were isolated and washed with deionized water several times and fixed with 2% glutaraldehyde solution at 4°C for 2 hrs followed by post fixing of specimen for 2 h with 1% osmium tetroxide solution, the samples were dehydrated with graded ethanol [18]. This sample was directly used for SEM and EDX analysis, gold and carbon coating was avoided. For HR-TEM dehydrated samples were sectioned using cryotome and then subjected to a carbon coated copper grid.

For confocal microscopy ZNPs were converted into its fluorescent counterpart by conjugation of rhodamine-B-isothiocyanate (RITC). Synthesis of RITC tagged ZNPs is described in paragraph below. A. niger cultured in potato dextrose broth (PDB) was incubated with 500 ppm of RITC tagged ZNPs for 72h and fungal samples were isolated and prepared using standard protocol as mentioned above[18]. Finally the samples were fixed with glue (Tissue-Tek, Sakura) and observed under confocal microscope.

(j) 2. Synthesis of RITC tagged ZNPs.
RITC tagged ZNPs were synthesized in two steps, first one involved the conversion of ZNPs into amine functionalized ZNPs while the second step involved conjugation of RITC to amine functionalized ZNPs.

- Synthesis of amine functionalized ZNPs:
Chapter 4

Amine functionalization of ZNPs was carried out by using 3-aminopropyltriethoxysilane (APTES) via co-condensation reaction using our previously reported method\textsuperscript{13}. In brief, about 0.5 g of ZNPs were dispersed in about 50 mL of DMSO in a sonication bath for about 1 hour. The resultant dispersion was transferred to a round bottom flask attached with a reflux condenser. To it 400 µL of APTES was added and the solution was refluxed at 120°C for about 3 hours. After completion of the reaction the resulting nanoparticle was centrifuged at 12000 rpm for about 15 minutes and washed several times with ethanol to remove the unreacted APTES. Finally the product was dried at 60°C overnight to produce amine functionalized ZnO nanorod.

- **Conjugation of RITC to amine functionalized ZNPs:**

ZNPs of appropriate concentration were dispersed in 0.1 M NaHCO\textsubscript{3} solution, to it 1 mg of RITC dissolved in 2 mL of aqueous DMSO (1:1, v/v) was added instantly and the reaction mixture was stirred at room temperature for 24 h in a dark condition. RITC labelled ZNPs (ZNPs-RITC) were separated by centrifugation at 10000 rpm at 4 °C. ZNPs-RITC were washed and re-dispersed in water repeatedly to remove excess of RITC.

#### 4.1.3. Results and discussions:

**(a) Mode of action study through determination of ROS:**

To delineate the mechanism of fungitoxic effect of ZNPs we performed biochemical analysis related to ROS on both strains of fungi. Interestingly dose dependent enhancement of ROS generation was observed in all of the biochemical tests. About 0.28 and 0.31 unit/mg protein/min SOD activity were found in control *A. niger* (MTCC 10180 and 2196 strains respectively). On the other hand 0.375 and 0.399 unit/mg protein/min SOD activity were found in 500 ppm ZNPs treated samples in same strains respectively. Dose dependent increase of SOD activity was also found in two strains of *F. oxysporum*. The function of SOD was mainly to scavenge ROS formed in living cells. Higher values of SOD activity at different ZNPs doses in comparison to that of control suggested that ZNPs induced the formation of superoxide and other ROS (figure 1a). Again, 0.25 and 0.23 unit/mg protein/min ascorbate peroxidase activity were found in MTCC 10180 and MTCC 2196 strains of *A. niger* respectively which was greater than the value of same in control. 0.32 and 0.28 unit/mg protein/min
ascorbate peroxidase activity were found in 500 ppm ZNPs treatment on the same strains respectively (figure 1b). Results of same assay in two strains of *F. oxysporum* also indicated increase in ascorbate peroxidase activity in treated cells (figure 1b). Even 0.1069 and 0.99 unit/mg protein/min catalase activity was found in MTCC10180 and MTCC 2196 strains of *A. niger* respectively which was also greater than control’s level. This activity was raised in 500 ppm treated fungal sample of same strains (0.1866 and 0.1820 unit/mg protein/min respectively) (figure 1c). Dose dependant increase of GR activity was sharply noticed in ZNPs treated all fungal strains with respect to control indicating higher enzyme activity either in response to the superoxide anions formed due to ZNPs activity against fungal call (figure 1d). The increase in intracellular thiol content with gradual increase in ZNPs concentration (figure 1e) indicated the role of thiol in detoxification of oxidative stress in all fungal strains. Malondialdehyde (MDA), the final product of lipid peroxidation, was found to be higher in 250 and 500 ppm treated samples when compared with control sets (figure 1f). Proline was a proteinogenic amino acid that served a variety of cellular functions and also detoxified ROS under stress [19]. At the highest dose (500 ppm ZNPs), proline was found to be constitutively higher in all fungal strains. It was speculated that proline levels were increased to detoxify ROS produced as a result of the stress generated by ZNPs (figure 2). Collectively these biochemical analyses revealed enhancement of ROS formation and subsequent increase in combating machineries to alleviate this ROS burden.
Figure 1. Comparative analysis of (a) Superoxide dismutase activity, (b) Ascorbate peroxidase activity, (c) Catalase activity, (d) Glutathione reductase (GR) activity, (e) Change in intracellular thiol content and (f) Extent of lipid peroxidation in fungal strains (*A. niger* and *F. oxysporum*) induced by two different concentrations (250 ppm and 500 ppm) of ZNPs respectively in comparison to control; where four fungal strains MTCC 10180 (*A. niger*), MTCC 2196 (*A. niger*), NCIM 1043 (*F. oxysporum*) and NCIM 1072 (*F. oxysporum*) were denoted by 1, 2, 3 and 4 respectively.
Figure 2. Change in proline content in fungal strains (A.niger and F. oxysporum) treated with different ZNPs concentrations (250 ppm and 500 ppm).
Figure 3. (a) Estimation of carbonyl content (as a marker of oxidative stress) of ZNPs treated fungal strains (*A. niger* and *F. oxysporum*) which resulted in protein damage, Where four fungal strains MTCC 10180, MTCC 2196, NCIM 1043 and NCIM 1072 were denoted by 1, 2, 3 and 4 respectively; (b) FTIR analysis of fungal cell (*A. niger*, MTCC 10180) :(A) Control (B) 250 ppm ZNPs treated (C) 500 ppm ZNPs treated; (c) Comparative study of gene expression of ZNPs and MZnO treated *A. niger* (MTCC 10180).

Here we have demonstrated carbonyl formation as an indicator of oxidative stress by DNPH binding assay as well as through FTIR studies. ROS could lead to side chain oxidation of protein and hence oxidized product was indexed as a carbonyl marker [20.21]. Total protein was estimated by using a standard procedure (Lowry’s Protocol). Post treatment increase in protein damage was observed for ZNPs at different concentrations (250 ppm and 500 ppm) after 48 h of inoculations in comparison with control samples (Figure 4a). Highest dose of ZNPs (500 ppm) produced 34248.73737 and 42272.72727 nanomol/mg of protein damage of cell membrane in case of MTCC 10180 and MTCC 2196 strains of *A. niger* respectively with respect to control, which produced only 25060.07362 nanomol/mg of protein damage. A remarkable dose dependent increase in carbonyl content was also detected in 250 ppm and 500 ppm ZNPs treated *F. oxysporum* strains (both in NCIM 1043 and NCIM 1072), indicating a hyper-oxidative state (figure 3a).

The damage sustained by fungal cell as a result of ZNPs action was indicated by FTIR study of fungus cell extract. Three bonds C-H, C-O and C-N were examined. The 2978 cm⁻¹ and 2900 cm⁻¹ peak, present in all the three samples is due to the C-H stretching frequency (figure 3b). The most intense signal for asymmetric and symmetric C-H stretching was obtained for 500 ppm ZNPs treated fungus sample (figure 3b(C)). ZNPs treatment on fungus triggered the rupture of cells thereby enhancing the modes of C-H stretching frequency. The 1645 cm⁻¹ band was the characteristic carbonyl stretching frequency which was present for all samples but the intensity was highest for 500 ppm treated sample. The most significant change in the FTIR spectra was in the region of 1045 cm⁻¹ which signified C-O stretching. The C-O signal intensity was lowest in the control sample (figure 3b (A)) and highest in 500 ppm ZNPs treated sample. C-N stretching frequency was registered at 1090
cm$^{-1}$ in all the three cases and the intensity was highest for 500 ppm ZNPs treated *A. niger*. Highest signal intensity of Zn-O stretching was observed at lower wavenumber 885 cm$^{-1}$ (figure 3b(C)) in the highest concentration of ZNPs treated fungus. The FTIR results demonstrated that 500 ppm ZNPs treated *A. niger* sample exhibited a remarkable increase in carbonyl content. This was a clear indication of hyper oxidative stress. ZNPs were capable of generating more reactive oxygen species such as superoxide radical, hydrogen peroxide and hydroxyl radical [21]. FTIR results validated our hypothesis that ROS formed by ZNPs lead to the oxidation of protein. The highest concentration of 500 ppm ZNPs in the series led to the highest ROS generation leading to most enhancements in signal intensity of carbonyl stretching frequency compared to control sample. Lin et al. [22] also observed this type of enhancement in C-O stretching frequency because of nanoparticle induced cell membrane rupture. However, we are the first to study mode of action of ZNPs on *A. niger* by estimating carbonyl content (as a marker of oxidative stress) through DNPH binding assay and FTIR analysis. Enhancement in carbonyl content in ZNPs treated fungus was confirmed by 12% SDS PAGE (figure 4) followed by western blot analysis (figure 5). These two figures clearly indicated that carbonyl content was distinctly increased in ZNPs treated sample in contrast to control. This outcome confirmed the enhancement of carbonyl content in treated sample as a result of ROS mediated damage which was mentioned previously.

![Figure 4](image_url)

**Figure 4.** SDS PAGE gel electrophoresis of control and ZNPs treated samples of *A. niger*. Increase of protein carbonylation (marker of oxidative stress) was shown in Lane 4 and 5 (250 ppm ZNPs treated
Chapter 4

*A. niger* sample) and Lane 7 and 8 (250 ppm ZNPs treated *A. niger* sample) with respect to control (Lane 1 and 2). Lane 3 and 6 denoted molecular marker (Protein marker, BLM003, 14-95 kDa, SRL).

![Image of gel electrophoresis](image)

**Figure 5.** Western blot analysis of control and ZNPs treated samples of *A. niger*. Immuno blot analysis of control and ZNPs treated (250 ppm and 500 ppm) *A. niger* samples with oxyblot detection kit (Millipore). Figure 3 (a) showed SDS PAGE of protein carbonylation (Lane 1, 2 denoted control, Lane 3, 6 denoted molecular marker, Lane 4, 5 denoted 250 ppm ZNPs treated *A. niger* sample and Lane 7, 8 denoted 500 ppm ZNPs treated *A. niger* samples). Figure 3 (b) showed corresponding western blot analysis of corresponding protein where Lane 1, 2 denoted control, Lane 3, 4 denoted 250 ppm ZNPs treated *A. niger* sample and Lane 5, 6 denoted 500 ppm ZNPs treated *A. niger* samples.

**(b) Microarray analysis:**

Our initial analysis determined the expression pattern of *A. niger* genes, directly or indirectly related to oxidative stress, were upregulated in ZNPs treated samples compared to control. We also compared the up regulation of genes in ZNPs with MZnO treated samples (figure 3c). The expression of genes related to alanine aspartate metabolism; amino acid biosynthesis and metabolism; carbohydrate metabolism; catalytic activity; electron transport; fatty acid biosynthesis; fatty acid metabolism; glutathione metabolism and biosynthesis; glycine, serine and threonine metabolism; lysine biosynthesis; methyl transferase; mitochondrial activity; oxidoreductase activity; pentose phosphate pathway; starch sucrose metabolism; superoxide activity; tryptophan activity were analyzed (figure...
4c). On an average up regulation of genes for nearly all the above described pathway was observed in case of ZNPs treatment in comparison with MZnO treatment. Electron transport, fatty acid metabolism, pentose phosphate pathway were the few exceptions.

Activity of alanine and aspartate metabolism should rise with the increase in oxidative stress [23]. Genes related to alanine and aspartate metabolisms were expressed 1.702 fold increase in ZNPs treated samples as compared with control ones. Amino acid biosynthesis and metabolism was important for sufficient growth and survival of fungus. Expression of genes related to these pathways was up regulated 1.942 and 2.1868 fold respectively which conveyed that regular amino acid biosynthesis and metabolism were impacted by stress. ZNPs, as an alpha amylase inhibitor has been reported by Dhobale et al. [24]. Expression of genes related to carbohydrate metabolism was 1.8729 fold up regulated in ZNPs treated cells. Glycine, serine and threonine metabolism also play a significant role in controlling oxidative stress [25]. Expression of genes related to glycine, serine and threonine metobolism was increased 2.307 fold. Expression of genes related to catalytic activity increased 2.064 fold in ZNPS treated A. niger which was an affirmation of our of enzyme assay results. Electron transport related genes were upregulated 2.1258 fold in ZNPs treated cells. Genes related to fatty acid biosynthesis and fatty acid metabolism were up regulated by 1.7814 and 2.64144 fold respectively in ZNPs treated fungus, which also complied with the result of lipid peroxidation [26]. Expression of genes related to glutathione biosynthesis and metabolism were raised 2.6515 fold which also supported the result of glutathione reductase assay. Lysine was a marker of oxidative stress [27], a 2.0872 fold increase was observed in ZNPs treated fungus which again supported our contention that ZNPs functioned as oxidative stress generators. Methyl transferase prevented apoptosis induced by oxidative stress [28, 29]. Mitochondrial activity [30] was also greatly affected by oxidative stress. 1.964 fold increase in mitochondrial activity related genes indirectly supported our hypothesis of oxidative stress generated by ZNPs. Oxidoreductase preeminently worked against oxidative stress [48] which concurred with the results of gene expression (2.355 fold increase) of oxidoreductase. Pentose phosphate an important pathway which regulated ROS generation [31] and the corresponding genes expressions were increased by 2.004 fold due to ZNPs treatment. Starch and
sucrose metabolism was directly related to pentose phosphate pathway which in turn was interlinked to oxidative stress generation [32] and corresponding over expressed genes in microarray results (1.715 fold increase) reflected the same. Gene expression related to superoxide dismutase was also enhanced 1.717333 fold thereby validating our previously described superoxide dismutase biochemical assay [33]. Tryptophan metabolism controller genes were also expressed 2.4855 fold increase compared to control. Tryptophan induced lipid peroxidation was a prominent marker of oxidative stress [34]. Therefore all these over expressed gene expression in microarray illustrated significant increase in ROS content by ZNPs (figure 3c). Over expressed genes in microarray analysis also correlated with the biochemical analysis, FTIR and gel electrophoresis results where oxidative stress was marked in terms of enhancement in carbonyl content. Moreover the up regulation in gene expression was significantly higher for ZNP's treatment in contrast to that of MZnO.

(c) Morphological study of fungal cell by SEM, TEM and AFM:

ROS generation and oxidative stress by ZNPs altered the morphology of fungal samples as expected. Smooth, prominent, continuous tube like structure was observed in the control samples of *A. niger* hypha (figure 6a) while 250 ppm (figure 6b) and 500 ppm ZNPs treated fungal hypha showed structural deformities in outer layer of cell wall under AFM which were most prominent in case of 500 ppm ZNPs treated sample (figure 6c). Healthy, uniform, regular, vasiform, uninterrupted cell surface of control hypha (figure 7a) under FE-SEM while ZNPs treated hypha (both 250 ppm and 500 ppm) showed dramatic change of hyphal cell surface. The treated samples had shriveled and inchoate morphologies (figure 7b and 7c). However number of ZNPs attached to fungal hypha was higher in case of 500 ppm treated fungal sample. HR-TEM micrographs provided a similar picture. Control fungal hypha of *A. niger* (figure 7d) contained highly dense cytoplasm, tightly attached within a thick cell wall. While ZNPs treated hypha (figure 7e and 7f) expressed cell deformation, entanglement and discontinuity of cell wall.
Figure 6. AFM study of Fungal hypha (*A. niger*, MTCC 10180): (a) Control (b) 250 ppm ZNPs treated (c) 500 ppm ZNPs treated.

Figure 7. Upper panel- FE SEM study of Fungal hypha (*A. niger*, MTCC 10180): (a) Control (b) 250 ppm ZNPs treated (c) 500 ppm ZNPs treated; Lower panel- Outer cell surface study of fungal hypha by HRTEM (*A. niger*, MTCC 10180): (d) Control (e) 250 ppm ZNPs treated and (f) 500 ppm ZNPs treated. Arrows in the lower panel demonstrated cell membrane damage.

(d) Zn$^{2+}$ release from ZNPs:

Release of Zn$^{2+}$ from ZNPs at the pH of PDA was evaluated by ICPMS shown in figure 8. Culture medium (PDA) had a pH of 5.5 - 6, therefore Zn$^{2+}$ release was measured at that particular pH 5.5 - 6. Interestingly control and 250 ppm ZNPs treated *A. niger* expressed more acidic pH in contrast to only
culture medium and 500 ppm treated *A. niger.* Acidic pH in control and ZNPs treated *A. niger* at low dose might be due to the release of secondary metabolites (toxin) from the fungal sample; while in high dose of ZNPs fungal growth was restricted which also resulted minimum release of secondary metabolites. Therefore pH in the highest dose was similar to that of culture medium. Hence, ZNPs acted as a small source of Zn$^{2+}$ which could simultaneously contribute to the fungitoxic effect of ZNPs as well. Similar Zn$^{2+}$ release from ZNPs was also evident by Wu et al. [35] and Arouja *et al.* [36] which promoted eco-toxicity to some extent.

*Figure 8.* Zn$^{2+}$ release from ZNPs by ICPMS analysis at three different interval of times 24h, 48h and 72h respectively. A small amount of Zn$^{2+}$ was released from ZNPs at the pH of fungal culture medium which could simultaneously contribute to the fungitoxic effect of ZNPs.

**(e) Bioavailability/uptake of ZNPs:**

Bioavailability/uptake of ZNPs to *A. niger* were clearly demonstrated by SEM analysis associated with EDX. About 13.69 wt % of Zn content was found to be present on the fungal hyphae (figure 79a). Conclusively HR-TEM image justified the presence of ZNPs on the fungal hyphae (figure 9b), the arrows illustrated the presence of ZNPs on the cross section of fungal hyphae. In contrast to the observation of Yan *et al.* [37] we found that the membrane of the fungal hyphae was ruptured.

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<th>Zn concentration (μM)</th>
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Biocidal mode of action study of ZNPs
loss of membrane integrity might be due to ROS generation from ZNPs. Once more crystalline structures of ZNPs were partly but still present after internalization to the fungal hyphae which was similar to the results obtained by Yan et al. [37, 38]. Partly crystalline nature of ZNPs on fungal hyphae was confirmed by selected area electron diffraction (SAED) pattern shown in figure 10.

**Figure 9.** (a) EDX analysis of ZNPs uptake by *A. niger* (MTCC 10180), inset illustrated corresponding SEM image, (b) HR-TEM image of ultrathin section of *A. niger* (MTCC 10180) hypha, where arrows demonstrated distribution of ZNPs on the fungal hypha.

**Figure 10.** Selected area diffraction pattern (SAED) of (a) ZNPs and (b) ZNPs after internalization on fungal hyphae. The first one i.e. native ZNPs exhibited distinct crystalline structure but after internalization on to fungal hyphae its crystalline pattern was partially lost but still present.
Figure 11. Confocal microscopic image of (a), (b), (c) Control *A. niger* (MTCC 10180) sample; (d), (e), (f) RITC tagged ZNPs treated *A. niger* (MTCC 10180) sample, where the later one clearly demonstrated bright red fluorescence at 540 nm excitation wavelength of RITC tagged ZNPs distributed on fungal sample.

When fungal sample was treated with RITC tagged ZNPs, a clear distinct distribution of RITC tagged ZNPs were observed on the fungal sample exhibiting bright red color at 540 nm excitation wavelength under confocal microscope (figure 7). But control sample did not exhibit any bright red fluorescence.
at all (figure 11). Therefore confocal microscopic analysis also revealed the bioavailability of ZNPs on the fungal sample.

4.1.4. Conclusion:

ZNPs absorb sufficient light/UV energy and result in the formation of electron- holes pairs through a process of electrostatic excitation between valence and conduction band. The generated electrons and holes undergo reaction with dissolved molecular oxygen, surface hydroxyl groups and absorbed water molecules to form hydroxyl (‘OH) and superoxide(O²⁻) radicals.

\[ e^+ + O_2 \rightarrow O_2^-; \quad O_2^- + H^+ \rightarrow HO_2^-; \quad HO_2^- + H^+ + e^- \rightarrow H_2O_2 \]

Enzyme assays revealed that ZNPs generated oxidative stress through ROS generation. In continuation it was shown that enhancement in carbonyl content was the marker of oxidative stress and its correlation with FTIR and microarray analysis was established. Presence of carbonyl content in ZNPs treated fungal samples was confirmed by gel electrophoresis and western blot analysis. Different micrographs (FE-SEM, HR-TEM, AFM) of fungal samples justified that ZNPs caused ROS mediated damage against fungus and ZNPs presence on the fungal sample was confirmed by EDX, HR-TEM and confocal microscopic analysis. All together we have demonstrated a total mode of action study for development of a fungicide using various biochemical and biophysical processes.

4.2. Bactericidal mode of action study:

4.2.1. Introduction:

To make better use of ZnO nanoparticles in food products and to assist in the development of powerful, but nontoxic, antimicrobial derivatives, it is necessary to understand the mechanism of action of ZnO nanoparticles against bacteria, but to date, the process underlying their antibacterial effect is not clear. However, a few studies have suggested that the primary cause of the antibacterial function might be from the disruption of cell membrane activity [39, 40]. Another possibility could be the induction of intercellular reactive oxygen species, including hydrogen peroxide (H₂O₂), a strong oxidizing agent harmful to bacterial cells [40, 41, and 42]. It has also been reported that ZnO can be activated by UV and visible light to generate highly reactive oxygen species such as OH⁻, H₂O₂, and
022'. The negatively charged hydroxyl radicals and superoxides cannot penetrate into the cell membrane and are likely to remain on the cell surface, whereas H2O2 can penetrate into bacterial cells [40,43]. To better understand the nature of the inhibitory and lethal effects of ZnO nanoparticles on bacteria, we used *E. coli* as a model organism to investigate this mechanism.

4.2.2. Experimental:

(a) Integrity of cell membranes:

If the bacteria membrane is compromised, release of cytoplasmic constituents of the cell can be monitored. Through the detection of absorbance at 260 nm, one can estimate the amount of DNA and RNA released from the cytoplasm. The experiments were conducted as follows. The bacterial suspension was separated into several flasks. Different concentrations of ZNPs were added to each flask except the control. Samples of 1.5 ml were removed from the flasks every 20 min. The samples were then immediately filtered with 0.2 mm syringe filters to remove the bacteria. The supernatant was then diluted appropriately and optical density at 260 nm was recorded [44].

(b) Outer membrane permeabilization assay:

Outer Membrane permeation activity of ZNPs was determined by the NPN (1-N-phenylamidnaphthylamine) assay [45]. *E. coli* (MTCC 443) cells grown in LB medium (to an optical density of A600 of 0.4) were collected, washed twice and resuspended in 5 mM sodium HEPES buffer, pH 7.2, containing 1 mM KCN. To 1 ml volume of bacteria in a quartz cuvette, NPN was added (final concentration: 10μM). Background fluorescence was recorded using a PerkinElmer LS 45 spectrophotometer (Perkin Elmer, UK). Excitation and emission wavelengths were set at 350 and 429 nm, respectively. ZNPs at different concentrations were added. Increase in fluorescence due to partitioning of NPN into the OM was recorded as a function of time until no further increase in intensity was recorded. Control tests were performed to verify that the enhanced fluorescence was due to NPN uptake by bacteria.

(c) Inner membrane permeabilization assay:

Inner Membrane permeation of *E. coli* (MTCC 443) was determined by measuring the release of β-galactosidase activity into the culture medium using ONPG (o-nitrophyl-β-D-galactoside) as a substrate [45]. Logarithmic-phase bacteria grown in LB broth containing 2% lactose were harvested,
washed, and resuspended in 0.5% NaCl solution. The final cell suspension was adjusted to obtain an A_{420} of 1.2. Different concentrations of ZNP (T) solutions (1.6 ml) were each mixed with bacteria suspension (1.6 ml) followed by 10 µL ONPG added to each samples. Plates were incubated with gentle rocking at 37°C and the production of o-nitrophenol as a function of cytoplasmic β-galactosidase release was monitored with a spectrophotometer at 420 nm over time.

(d) Cell morphology study by SEM:

For Scanning Electron Microscopic (SEM), after 24 h of inoculation, bacterial samples (E.coli, control and ZNPs treated) were isolated and prepared following standard procedure [16].

4.2.3. Results and Discussions:

(a) Integrity of bacterial cell membranes:

The cytoplasmic cell membrane is a structural component, which may become damaged and functionally invalid when bacterial suspensions are exposed to antibacterial agents. If bacterial membrane become compromised, small ions such as K⁺ and PO_{4}^{3-} tend to leach out first, followed by large molecules such as DNA, RNA and other materials. The release of intracellular components with strong UV absorption at 260 nm is an indication of membrane damage [44]. When E. coli suspensions were treated with ZNP (T), the A_{260} increased rapidly at first then at a decreasing rate up to 180 min (figure 12a). Thus, the damage of cell membranes by ZNP (T) is concentration dependent, which is agreeable with the findings for bactericidal activity.

![Absorbance at 260 nm](image1)

![PL intensity vs Wavelength](image2)
Figure 12. (a) Cell membrane integrity assay by DNA release study; (b) Outer membrane permeability assay of control and ZNPs treated *E. coli* (MTCC 443).

(b) Outer membrane permeabilization assay:
Gram negative bacteria such as *E. coli* have two cell envelope membranes. The ability of ZNP(T) to interact with both outer and inner membranes were examined. NPN, which is a hydrophobic probe is normally excluded by an intact outer membrane of gram negative bacteria. Increased fluorescence uptake of NPN occurs in bacterial suspensions containing cells whose outer membranes are damaged and functionally invalid, since the quantum yield of NPN is greatly increased [45]. The addition of ZNP (T) to *E. coli* suspensions in the presence of NPN caused a time-dependent increase in fluorescence (figure 12b). The relative fluorescence increased to a maximum in 10 min. The maximum fluorescence was greater with the higher than the lower concentration of ZNP(T). These results suggested that ZNP (T) rapidly increase the permeability of the outer membrane of cells.

(c) Inner membrane permeabilization assay:
When the inner membrane became compromised, β-galactosidase, a normally endoenzyme could permeate the cytoplasmic membrane. The ability of ZNP (T) to permeate *E. coli* inner membrane was evaluated by using the production of cytoplasmic β-galactosidase with bacteria grown in lactose containing media. When *E. coli* suspensions were treated with ZNP (T), there was immediate release of β-galactosidase with the amount released being maximal after about 60 min (figure 13). For untreated suspensions, there was a lag of about 30 min before β-galactosidase was released relatively slowly. The increased release of cytoplasmic β-galactosidase caused by ZNP (T) indicates that the permeability of IM had increased, which is agreeable with the other findings for *E. coli* cells that were not dividing.
Figure 13. Inner membrane permeability assay of ZNPs treated E.coli (MTCC 443) compared to control.

(d) Cell morphology study by SEM:
Healthy, uniform, regular, uninterrupted cell surface of control hypha (figure 14) under FE-SEM while ZNPs treated E. coli (200 µg/mL) expressed cell deformation and discontinuity of cell wall.

Figure 14. FESEM image of control (a) and ZNPs treated E.coli (MTCC 443) (b).

4.2.4. Conclusion:
We have studied antibacterial activity of ZNPs. It can be concluded that the cell wall rupture must be due to the surface activity of ZNPs in contact with the bacteria. It is believed that cell death is caused by the decomposition of the cell wall followed by the subsequent decomposition of the cell membrane. The damage to the cell membrane directly leads to the leakage of minerals, proteins and
genetic materials, causing cell death. ZNPs has a band gap of 3.37ev (Figure 15) [46]. So photon energy equal or more than 3.37ev results in the transmission of electron from the valence bond (VB) to conduction bond (CB). This causes region of positive charges ($h^+$) at VB and a free electron ($e^-$) in CB. At the ZNPs surface, the holes react with surface hydroxyl group (OH) and adsorbed $H_2O$ molecules to form $OH^-$ radicals. In the absence of electron acceptors the electron hole recombination is possible. The presence of oxygen prevents this recombination by trapping electron and form superoxide ions. The final product of the reduction may also be $OH^-$ radicals and the hydroperoxy radical $HO_2^-$. Hydroxyl radicals have the power to oxidise the organic compounds absorbed on to the semiconductor surface and inactivate microorganism.

![Diagram of band gap of ZNPs]

**Figure 15: Band gap of ZNPs**

\[
\text{ZnO + photon} \rightarrow h^+ + e^-
\]

\[
H_2O \rightarrow H^+ + OH^-
\]

\[
h^+ + OH^- \rightarrow OH^-
\]

\[
h^+ + H_2O \rightarrow H^+ + OH^- / HO_2^-
\]

\[
e^- + O_2 \rightarrow O_2^-
\]

\[
HO_2^- + H^+ + e^- \rightarrow H_2O_2
\]

\[
2 O_2^- + H^+ \rightarrow 2OH^- + O_2
\]

\[
\text{Organics} + OH^- + O_2 \rightarrow CO_2 + H_2O + \text{etc}
\]
Since, the hydroxyl radicals and superoxides are negatively charged particles, they cannot penetrate into the cell membrane and must remain in direct contact with the outer surface of the cell, however H$_2$O$_2$ can penetrate into the cell [47]. The smaller particle size shows enhanced activity due to the large surface area to volume ratio and the surface reactivity of nano ZnO. The surface area to volume ratio of nano ZnO is higher than bulk ZnO. The generation of H$_2$O$_2$ depends strongly on the surface area of ZnO, which results in more reactive oxygen species on the surface and thus the higher antimicrobial activity of smaller nanoparticles.
Chapter 4

References:


Chapter 4


Chapter 4


Biocidal mode of action study of ZNPs


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Biocidal mode of action study of ZNPs
Chapter 4


