The effect of zinc oxide nanoparticles against ceiling mold isolate

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Abstract. In the current study, the antifungal activity of zinc oxide nanoparticles (ZnO NPs) against the ceiling mold isolates Penicillium sp. A1P and Cladosporium sp. A2C was examined. Antifungal activity was tested using Potato Dextrose Agar (PDA) plates supplemented with 0.1 %, 0.25 %, 0.5 % or 1.0 % (w/v) ZnO, and the growth of mold isolates on the supplemented media was observed using the slide culture method. Minimum Fungal Concentration (MFC) was determined by tube dilution method using Potato Dextrose Broth (PDB) supplemented with 0.5 %, 1.0 %, 1.5 % or 2.0 % (w/v) ZnO NP concentration. MFC was determined on the basis of Total Plate Count (TPC) obtained from tube dilution. Results from plate agar and slide cultures showed that ZnO NPs have fungistatic effects that increased with increasing ZnO NP concentration. Penicillium sp. A1P and Cladosporium sp. A2C remained biologically active; however, the spores germinated relatively slow and the diameters of resulting colonies decreased. The growth of mold isolates in supplemented broth media was notably slower than in the control, supporting the data from the plate agar and slide culture experiments. In conclusion, ZnO NPs have fungistatic against the ceiling mold isolates.

Keywords: Antifungal, Cladosporium, Penicillium, zinc oxide nanoparticles

1. Introduction
Certain nanoparticles (NPs) are used in various industries because of their antimicrobial properties. Zinc Oxide (ZnO) NPs, for example, have been utilized as antifungal agents; many previous studies have shown that ZnO NPs inhibit the growth of fungi such as Aspergillus niger, Penicillium chrysogenum and Fusarium oxysporum [1]. In Indonesia, fungal biodegradation occurs in many materials, often because of high humidity. As a result, the use of nanoparticles for fungal control may alleviate the economic costs associated with fungal biodegradation. The aim of the current study is to determine the effect of ZnO NPs on the ceiling mold isolates Penicillium sp. A1P and Cladosporium sp. A2C, and the concentration of ZnO NPs required for inhibiting fungal growth.
2. Materials and method

2.1. Materials
Penicillium sp. A1P and Cladosporium sp. A2C were supplied by Lab. Microbiology, Dept. Biology, FMIPA-UI. ZnO NPs were purchased from US Research Nanomaterials, Inc., Houston, TX 77084, USA. Media Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) were used for maintenance the mold culture and antifungal assay.

2.2. Fungal spore preparation for enumeration
Fungal spore enumeration was prepared by growing Penicillium sp. A1P and Cladosporium sp. A2C isolates on PDA slant medium for 7 days. The spores were suspended in sterilized aquadest and diluted up to $10^{-7}$. Enumeration was done using Total Plate Count (TPC) method and started from $10^{-4}$ to $10^{-7}$ dilution. Each plates was inoculated with 0.1 mL of spore suspension from the dilution and each dilution was done in three replication. All inoculated plates were incubated at 30 °C for 48 hours. The total number of colony-forming units (CFU) was calculated based on the following formula:

$$CFU = \frac{\sum \text{colonies}}{\text{dilution factor} \times \text{volume inoculum}}$$

2.3. Antifungal assay
Antifungal activity of ZnO NPs was studied using PDA plate method and slide culture method; for each technique, media was supplemented with 0.1 %, 0.25 %, 0.5 %, 1.0 %, 1.5 % and 2.0 % (w/v) ZnO NPs. PDA without ZnO NPs was used as control. Each medium was inoculated with Penicillium sp. A1P and Cladosporium sp. A2C spores, and plates were incubated at 30 °C. Growth was observed daily for 7 days.

2.4. Determination of the minimal fungicidal concentration
Determination of the Minimal Fungicidal Concentration (MFC) was performed by tube dilution method. PDB was supplemented with 0.1 %, 0.25 %, 0.5 %, 1.0 %, 1.5 % and 2.0 % (w/v) ZnO NPs. Each tube was inoculated with a final spore concentration of $10^7$ CFU/mL. All cultures were incubated at room temperature and observed daily for 7 days. After 7 days of incubation, the fungicidal effects of ZnO NPs were evaluated at each concentration using the total plate count (TPC). The percent survival rates were calculated using the following formula:

$$\% \text{Survival rate} = \frac{\sum \text{survival colonies}}{\sum \text{initial colonies}} \times 100 \%$$ (1)

3. Results and discussion

3.1. Spore enumeration
Result of enumeration showed that spore population of Penicillium sp. A1P (table 1) and Cladosporium A2C (table 2) were almost the same, about $10^7$ CFU/mL. Images of the TPC results from Penicillium sp. A1P and Cladosporium sp. A2C are shown in figure 1.

3.2. Antifungal assay: plate agar method
The growth of Penicillium sp. A1P and Cladosporium sp. A2C isolates on ZnO NP supplemented PDA indicated that the ZnO NPs inhibited fungal growth compared to the control. This inhibition resulted in reduced diameter colony of Penicillium sp. A1P (figure 2) and Cladosporium sp. A2C (figure 3), after 7 days of incubation. The ZnO NP was also caused late germination, sporulation, and reduced number
of *Penicillium* sp. A1P spores (table 3) and *Clostridium* sp. A2C spores (table 4) qualitatively compared to control.

### 3.3. Antifungal assay: slide culture method

Since the fungal observation can be done directly under microscope, the slide culture method were taken to support the Plate Agar method. The result on the slide culture showed the germination and sporulation were relatively slower (table 5 and table 6), and the colonies’ diameter after 7 days incubation were also smaller than the control (figure 3). These results are similar to those of the plate agar method.

### 3.4. Minimal fungicidal concentration

The growth observation of *Penicillium* sp. A1P and *Cladosporium* sp. A2C in PDB medium controle showed that hyphae formation appeared on 2nd day for *Penicillium* sp. A1P and 3rd day for *Cladosporium* sp. A2C. On the other hand, the hyphae formation did not appear until the 7th day of observation, in all PDB media added with ZnO NPs (figure 4). If the appearance of fungal growth was an indicator of fungicidal’s effect, so those result indicated that the MFC of *Penicillium* sp. A1P and *Cladosporium* sp. A2C were lower or equal to 0.5 %. The result was paralleled with the research conducted by He et al. [2] and Singh et al. [3].

| Table 1. Results of total plate count from the isolate *Penicillium* sp. A1P. |
| --- |
| Dilution factor | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
| Replication | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| Σ Colonies | > 300 | > 300 | > 300 | 73 | 71 | 58 | 9 | 5 | 7 |
| Average (CFU/mL) | > 300 $\times 10^{5}$ | 6.8 $\times 10^{7}$ | 7.0 $\times 10^{7}$ |

| Table 2. Results of total plate count from the isolate *Cladosporium* sp. A2C. |
| --- |
| Dilution factor | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
| Replication | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| Σ Colonies | > 300 | > 300 | > 300 | 32 | 55 | 41 | 5 | 3 | 2 |
| Average (CFU/mL) | > 300 $\times 10^{5}$ | 4.3 $\times 10^{7}$ | 3.3 $\times 10^{7}$ |

Figure 1. Total Plate Count of isolates: (a) *Penicillium* sp. A1P, (b) *Cladosporium* sp. A2C.
Figure 2. Diameter growth average of *Penicillium* sp. A1P.

Figure 3. Diameter growth average of *Cladosporium* sp. A2C.

Table 3. Growth of fungal isolate *Penicillium* sp. A1P.

| [Conc.] of ZnO NPs (%) | Average Ø colonies (cm) | Germination (day) | Sporulation (day) | Σ Spora | Growing zone |
|------------------------|-------------------------|-------------------|-------------------|---------|--------------|
| 0                      | 1.92                    | 1<sup>st</sup>    | 2<sup>nd</sup>    | ++++    | √            |
| 0.1                    | 0.98                    | 1<sup>st</sup>    | 3<sup>rd</sup>    | +++     | √            |
| 0.25                   | 0.73                    | 1<sup>st</sup>    | 4<sup>th</sup>    | +       | √            |
| 0.5                    | 0.53                    | 2<sup>nd</sup>    | 4<sup>th</sup>    | +       | √            |
| 1.0                    | 0.38                    | 2<sup>nd</sup>    | 4<sup>th</sup>    | +       | √            |
### Table 4. Growth of fungal isolate *Cladosporium* sp. A2C.

| [Conc] of ZnO NPs (%) | Average Ø colonies (cm) | Germination (day) | Sporulation (day) | Σ Spora | Growing zone |
|-----------------------|--------------------------|-------------------|-------------------|---------|--------------|
| 0                     | 1.93                     | 1st               | 3rd               | ++++    | ı             |
| 0.1                   | 0.56                     | 1st               | 4th               | +       | ı             |
| 0.25                  | 0.36                     | 1st               | 4th               | +       | ı             |
| 0.5                   | 0.30                     | 1st               | 4th               | -       | ı             |
| 1.0                   | 0.30                     | 2nd               | 5th               | -       | ı             |

### Table 5. Fungal growth of isolate *Penicillium* sp. A1P at 7 days incubation.

| [Conc] of ZnO NPs (%) | Average Ø colonies (µm) | Germination (day) | Sporulation (day) | Ø Hyphae (µm) |
|-----------------------|--------------------------|-------------------|-------------------|---------------|
| 0                     | 1429                     | 1st               | 2nd               | 4.0           |
| 0.1                   | 1036                     | 1st               | 2nd               | 4.0           |
| 0.25                  | 973                      | 1st               | 2nd               | 4.0           |
| 0.5                   | 914                      | 1st               | 3rd               | 4.0           |
| 1.0                   | 835                      | 2nd               | 3rd               | 3.5           |
| 1.5                   | 741                      | 3rd               | 6th               | 3.5           |
| 2.0                   | 659                      | 3rd               | 6th               | 3.5           |

### Table 6. Fungal growth of isolate *Cladosporium* sp. A2C at 7 days incubation.

| [Conc] of ZnO NPs (%) | Average Ø colonies (µm) | Germination (day) | Sporulation (day) | Ø Hyphae (µm) |
|-----------------------|--------------------------|-------------------|-------------------|---------------|
| 0                     | 1237                     | 1st               | 3rd               | 4.0           |
| 0.1                   | 1118                     | 1st               | 4th               | 4.0           |
| 0.25                  | 993                      | 1st               | 4th               | 4.0           |
| 0.5                   | 914                      | 1st               | 4th               | 4.0           |
| 1.0                   | 837                      | 2nd               | 5th               | 3.5           |
| 1.5                   | 752                      | 3rd               | 5th               | 3.5           |
| 2.0                   | 693                      | 3rd               | 5th               | 3.5           |

**Figure 4.** Growth of isolate at 7th day of observation: (a) *Penicillium* sp. A1P, and (b) *Cladosporium* sp. A2C isolate.
Table 7. Survival rate of isolate *Penicillium* sp. A1P.

| [Conc] of ZnO NPs | 0.5% | 1.0% | 1.5% | 2.0% |
|------------------|------|------|------|------|
| Replication      | 1    | 2    | 3    | 1    |
| Σ Colonies       | >>>  | >>>  | >>>  | 127  |
| Average (CFU/mL) | > 250| 145  | 42   | 21   |
| Survival cell/mL | > 2500| 1450 | 420  | 210  |
| % Survival rate  | > 2.5| 1.45 | 0.42 | 0.21 |

Table 8. Survival rate of isolate *Clostridium* sp. A2C.

| [Conc] of ZnO NPs | 0.5% | 1.0% | 1.5% | 2.0% |
|------------------|------|------|------|------|
| Replication      | 1    | 2    | 3    | 1    |
| Σ Colonies       | 115  | 147  | 120  | 145  |
| Average (CFU/mL) | 127  | 149  | 145  | 35   |
| Survival cell/mL | 1270 | 1490 | 1450 | 350  |
| % Survival rate  | 1.27 | 1.49 | 1.45 | 0.35 |

In the other hand, the result of TPC from PDB medium added with ZnO NPs still showed fungal growth. The percentage of survival rate of *Penicillium* sp. A1P at 2% ZnO NPs was 0.21% and *Cladosporium* sp. A2C was 0.35% (table 7 and table 8). Those results were still higher than the percentage of survival rate limit of fungicidal effect. According to Hugo et al. [4], the fungicidal effects are defined as the destruction of 99.9% of initial spores or only 0.1% cells will survive.

4. Conclusion
Although there was no fungal growth after 7 days incubation in PDB medium with 0.5% ZnO NPs, however ZnO NPs did not have fungicidal effects. It seemed ZnO NPs was fungalstatic rather than fungicidal, because it cannot kill 99.9% of initial inoculum of *Penicillium* sp. A1P and *Cladosporium* sp. A2C isolate.

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