Antibacterial activity of copper oxide nanoparticles prepared by mechanical milling

M I Amal1*, J T Wibowo2, L Nuraini1, G Senopati1, M Y Hasbi1, G Priyotomo1
1 Research Centre for Metallurgy and Materials, Indonesian Institute of Sciences, Tangerang Selatan, Indonesia
2 Research Centre for Oceanography, Indonesian Institute of Sciences, Jakarta, Indonesia

*E-mail: muha137@lipi.go.id

Abstract. In this study, Cu2O (copper oxide) nanoparticles were synthesized by using mechanical milling and tested for antibacterial properties. The raw material for copper oxide powder (macroparticles) was refined in size using an instrument of shaker mill with a 1: 1 ball and powder ratio and a time variation of up to 16 hours. X-Ray Diffraction (XRD) diffraction data showed changes in crystallinity and size depending on the grinding time from the calculation of the crystallite size. The results of microstructure observation using an electronic microscope (Scanning Electron Microscope, SEM) also showed changes in particle size correlated with the length of the milling process. Antibacterial activity was carried out on the Vibrio eltor, Bacillus subtilis, Staphylococcus aureus, Candida tropicalis, and Saccharomyces cerevisiae with the effect of particle size on antibacterial activity is discussed.

1. Introduction
The group of metal oxides compound is well-known to have wide range of applications in addition of their abundance in nature and easiness of synthesis. Copper oxide, in particular, has been studied for its potential as an antifouling agent, antibacterial active agent, pesticide, photocatalyst, solar cells, pigments, flame retardants, and chemotherapeutic agent [1–7]. The functional properties of materials are generally enhanced when it comes into nanoscale. Generally, two approaches have been introduced to prepare copper oxide nanoparticles, i.e. top-down and bottom-up that apply physical and chemical process or combination of both. Physical vapor deposition (PVD), chemical vapor deposition (CVD), sol-gel synthesis, sonochemistry, solvothermal, and mechanochemical synthesis are among the methods. One emerging technique that employs biological entity as natural biofactories for producing nanoparticles has also been introduced recently [8]. Aforementioned techniques to produce copper oxide nanoparticles have their own benefit and drawback, thus careful selection of appropriate technique to prepare copper oxide nanoparticles that meets designated functional properties is a must.

The application of copper oxide as antifouling agents has been introduced long time ago. However, there is a raised concern regarding the toxicity of copper oxide that can be harmful to other marine biota. Some countries such as Sweden and Netherlands have restricted the use of copper-containing antifouling paints, though in Indonesia it is still used widely. Some findings suggest that the risk of copper oxide harmful depends on the marine environment. One way to reduce the risk is by decreasing the amount of copper oxide in the marine coating while preserve its effectivity. It can be done by
refining the size into nanoscale, thus increasing the active surface area and functional antifouling activity. Other benefits of nanoparticle are high compatibility to various organic and inorganic polymer matrix as well as good stability at high temperature.

In this study, copper oxide nanoparticle was prepared by applying mechanical milling technique, a physical top-down approach. This method is known for its efficient cost and easiness to scale-up, while it can be detrimental to some materials, especially organic compounds. The structural properties, morphology and particle size of refined powders were analysed. Finally, antibacterial activity was assessed using agar diffusion method and its correlation of effectiveness to particle size was studied.

2. Experimental

The type of mechanical milling applied in this experiment was a high energy ball mill designed by Research Center for Physics, Indonesian Institute of Sciences. The milling process was interrupted in 3 minutes interval and continued after 2 minutes stopping to avoid temperature elevation. The Ball-to-Powder Ratio (BPR) was 1:1 with the diameter of alumina ball was 1 mm. In order to investigate the effect of mechanical milling process to the particle size, the duration of milling was varied from 4 to 16 hours. The raw material was commercially available with initial particle size of 2−5 µm (Sigma-Aldrich).

The as-milled copper oxides were characterized for its structural properties by using X-Ray Diffraction (XRD; Miniflex Rigaku). The visual observation was assisted by using Scanning Electron Microscope (SEM) attached with Energy Dispersive X-ray Spectrophotometer (EDX) (JEOL JSM-6390A) to analyze its composition. Particle Size Analyzer (PSA; LS 200 Beckman Coulter) was used to determine the particle size distribution of as-milled copper oxide particles. The qualitative antibacterial assay of the as-milled copper oxides powder was carried out using agar diffusion method against *Vibrio eltor*, *Bacillus subtilis*, and *Staphylococcus aureus* with methanol (Bratachem) as positive control. The qualitative antibacterial assay procedure is reported elsewhere [9].

The effect of particle size to antibacterial activity of refined copper oxides particle was investigated against *Candida tropicalis* and *Saccharomyces cerevisiae* with n-hexane (Merck) as positive control. Firstly, extract of inorganic compound with a concentration of 2.4 mg/ ml using 20% n-hexane as a solvent was prepared. Culture of microbial targets, i.e. *Candida tropicalis* and *Saccharomyces cerevisiae* was diluted 10 times. 100 µl of Yeast Mannitol Broth (YMB) (Sigma-Aldrich) media is inserted in the microplate of the first column to the third column. The first column was containing the media where 100 µl of 2.4 mg/ml concentration test was added, and then the mixture was homogenized by stirring using a micropipette. Following the homogenization, the dilution was inserted by the media mixture and the test extracts for the first column into the second column and then was homogenized. The dilution was processed until the third column, so that the desired final concentration is 1.2, 0.6, and 0.3 (µg/ml). Each column that has contained a mixture of media and extract with various concentrations was deposited with 10 µl of target microbial suspension (C. tropicalis) with a ten times dilution. Furthermore, the mixture was incubated using a shaker with room temperature for 24 hours. Yeast growth was observed by looking at the turbidity. The mixture was measured with a microplate reader at a wavelength of 595 nm. The same treatment was carried out for S. cerevisiae target microbes with similar ten times of dilution, and n-hexane solvent was used comparison (positive control).

3. Results and Discussions

Figure 1 shows the morphology of copper oxides after refinement process by high energy shaker mill up to 16 hours. All SEM images showed inhomogeneous distribution of particle from tens of micrometer to sub-micron of size. This finding is common for mechanomilling results, as the powders can be agglomerated in some extent. Yet, the pulverization resulted to powder refinement which increased as longer milling applied.
Figure 1. Scanning electron micrographs of as-milled copper oxides powder after 4, 8, 12, and 16 hours for a), b), c), and d) respectively.

The semi quantitative EDX analysis revealed the presence of copper and oxygen without any impurities as shown in Figure 2. The atomic percentage ratio indicated a Cu$_2$O phase except for samples of 12 and 16 hours milling, having a slight distortion due to the presence of CuO.

Figure 2. EDX spectra of as-milled copper oxides powder after 4 hours. The other samples also showed the similar result.

X-Ray diffractogram of samples in Figure 3 also confirmed the phase formation of these copper oxides compounds. The peaks at 29.5, 36.5, 42.3, 61.4, and 73.5$^\circ$ referred to Cu$_2$O phase in accordance to ICSD 00–005-0667 (copper oxide). The as-milled powders of 12 and 16 hours showed additional peak at 38.7$^\circ$ that fitted to copper dioxide peak of ICSD 00–045-0937. The evolution of some Cu$_2$O into CuO is possible due to extensive process of oxidation and more generated thermal energy after longer milling hours. XRD spectra also show that crystallinity of samples was decreased.
toward increasing of milling time. The change in crystallinity was affected by the refinement of powder as well as the deformation of crystal structure due to collision of ball milling.

Figure 3. XRD spectra of Cu$_2$O powders with different milling hours. As-milled powders of 12 and 16 hours also contained CuO phase.

Figure 4. Crystallite size and average of particle size of samples.
Figure 4 shows the change of average particle size and crystallite size of samples after milling. The average particle size was obtained from particle size analysis result while crystallite size was calculated from XRD data by Scherrer equation. Both particle and crystallite size have straightforward correlation to the mechanical milling process, which longer milling time gave finer particle size. The discrepancy between average particle size and crystallite size became smaller as milling time increased. Eventually, a particle was equivalent to single crystallite after refinement process of 12 hours, while before that the particles were either agglomeration of small particles or single big particle. The average particle size was decreased from around one micrometre of original particle size before milling to around 110 nm after 16 hours milling. The physical refinement method of mechanical milling is difficult to reach particle size under 100 nm due to limitation of ball size. To obtain particle size below 100 nm, it is needed extremely small size of milling balls.

Table 1. Qualitative antibacterial assay using as-milled Cu$_2$O after 16 hours and methanol as control. (+) indicates activity, while (-) indicates inactivity.

| Sample      | Vibrio eltor | Bacillus subtilis | Staphylococcus aureus |
|-------------|--------------|-------------------|-----------------------|
| 24 hours incubation with Cu$_2$O concentration of 1 mg/mL |              |                   |                       |
| Cu$_2$O     | +            | +                 | +                     |
| Methanol    | -            | -                 | -                     |
| 72 hours incubation with Cu$_2$O concentration of 1 mg/mL |              |                   |                       |
| Cu$_2$O     | +            | +                 | +                     |
| Methanol    | -            | -                 | -                     |

Generally, bacteria are distinguished based on the difference in the structure of bacterial wall. Gram-negative bacteria have an outer cell membrane in contrast of gram-positive bacteria. Yet, the cell wall of gram-positive bacteria is rich of peptidoglycan. To examine the antibacterial activity of as-milled copper oxides, gram-negative bacteria of V. eltor and gram-positive of B. subtilis and S. aureus were used. Table 1 shows the qualitative antibacterial activity assay using as-milled Cu$_2$O after 16 hrs and methanol as control. The inhibition zone was observed after 24 and 72 hours incubation. The antibacterial activity indicated that the as-milled have activity toward bacteria compared to methanol. The antibacterial mechanism depends on the bacteria type, i.e. gram-negative or gram-positive. The oxidation of microbial surface molecules due to the ionic interaction between positive charge of nanoparticles and negative charge of microbe, will lead to microbes death. While in the gram-positive bacteria, the antibacterial mechanism is related to cell wall biodestruction through degradative effects of DNA [10]. The qualitative antibacterial assay suggested the potency of copper oxide nanoparticle as antibacterial agent. Based on this, the quantitative analysis was carried out to study the antibacterial activity efficiency of copper oxides nanoparticles toward C. tropicalis and S. cerevisiae. C. tropicalis is a species of yeast and a common pathogen in neutropenic hosts. While, S. cerevisiae is the only yeast cell that has Berkeley bodies, a unique organelle that involves in particular secretory pathways.

Figure 5 shows the antibacterial activity examination against C. tropicalis and S. cerevisiae. It is obvious that particle size affected the surface area and the ability to continuously release copper ions in solution. Hence, the interaction of copper ions toward microbial cell wall would be more intense if the particle size is smaller. In addition, both Cu$^+$ and Cu$_2$O$^+$ ions have antimicrobial effect that only
differs in mechanism. Thus the presence of different copper oxides in samples 12 and 16 hours did not influence the antimicrobial activity efficiency. The results of antibacterial activity showed similar tendency in as-milled powders of 8, 12, and 16 hours. It hinted that to increase antibacterial activity, the particle size is needed to be below 100 nm.

![Figure 5. Antibacterial activity examination using as-milled CuO with different milling hours against C. tropicalis and S. cerevisiae.](image)

4. Conclusion
The nanosized copper oxides were successfully prepared by cost-effective and simple method of mechanical milling. Through 16 hours of milling by using high-energy shaker mill, the particle refinement showed 90% reduction from initial particle size. Both the semi quantitative analysis of EDX and structural analysis of XRD depicted the presence of only copper oxides compound. Yet, in as-milled powders of 12 and 16 hours, the copper oxide phases were CuO and small presence of Cu$_2$O. The antibacterial activity was performed against *Vibrio eltor*, *Bacillus subtilis*, and *Staphylococcus aureus* with methanol as positive control for qualitative assay and *Candida tropicalis* and *Saccharomycetes cerevisiae* for quantitative assay. The result showed the effectivity of nanoparticle copper oxides as biocidal agent. Furthermore, it also indicated that smaller the particle size, the higher the efficiency of antibacterial activity.

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