DOI: 10.21776/ub.jpacr.2021.010.03.594 *J. Pure App. Chem. Res.*, **2021**, 10 (3), 165-174
05 November 2021

Biosynthesis of Cu_2O/CuO-NP and Ag-NP Using *Rhizopus oligosporus* as Reductor Agent

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Received 17 May 2021; Accepted 05 November 2021

ABSTRACT

Nanoparticles (NP) have been widely used in various fields which depends on its size and shape. Nanoparticles can be synthesized physically and chemically. However, these methods need large amount of energy, not environmental friendly and quite expensive, because requires several additional materials besides precursors. In this study, we performed the analysis of some parameters to determine the best conditions for biosynthesis using *Rhizopus oligosporus* to obtain the nanoparticles with specific size. This fungi was used because easy to find, cultivate, and handle. We found that grain size of Cu_2O/CuO-NP and AgNP obtained are 23 nm, 55 nm respectively. In conclusion, this study confirms that some parameters investigated and *Rhizopus oligosporus* can be used to obtain particle in specific size.

Keywords: biosynthesis, Cu_2O/CuO-NP, AgNP, *Rhizopus oligosporus*

INTRODUCTION

Silver nanoparticles (AgNP) and Copper Nanoparticles (CuNP) have been widely recognized as being useful in various fields such as disinfectants for medical equipment and anti-microbial pathogens, wound healing, biosensor materials, wearable devices. These materials can be synthesized physically, chemically, or biologically. In physical method, a large amount of energy is required to create a constantly increasing temperature around the source material and it takes a long time to stabilize the temperature [1]. Meanwhile, the chemical method is not environmental friendly and requires several additional materials besides precursors. The additional materials are very important in synthesis process. The protective agent used to stabilize the dispersed nanoparticles during the formation of nanoparticles, and to protect the nanoparticles that may be absorbed or form bonds on the surface of the nanoparticles, which can inhibit the agglomeration process [2]. Thus several studies mainly focused on developing other methods to reduce their impact on the environment and lower energy. Biosynthesis is considered method which can reduce the use of reagents and expected to be more environmental friendly. The reducing agents that have been studied are extracts of plants, bacteria, and fungi. Apart from the type of reducing agent, factors that influence the biosynthesis such as temperature and stirring time may result in different shapes and sizes of the nanoparticles.

Various research results indicate that biosynthesis with different microorganisms and biosynthetic conditions may result different size and shape of nanoparticles which is reasonable because of the compounds contained [3]. Some fungi that have been studied for the biosynthesis of AgNP are *Saccharomyces cerevisiae*, *Rhizopus stolonifer*, and *Aspergillus flavus NJP08*. AgNP

which is formed in biosynthesis using *Rhizopus stolonifer* has a spherical mono dispersion shape with a diameter of 9.47 nm [4]. Meanwhile AgNP which synthesized with *Saccharomyces cerevisiae* produces a spherical shape with a diameter of 5.4 nm [5]. While the AgNP formed from biosynthesis using *Aspergillus flavus NJP08* produces a spherical shape with a diameter of 17 ± 5.9 nm [6].

In this study, Cu_2ONP, CuONP, and AgNP biosynthesis was carried out using *Rhizopus oligosporus* which has not been used in recent studies. This fungi has been widely used by Indonesian for tempeh productions. This fungi is produced commercially in inoculum form thus easier for people to make tempeh and the price is quite affordable. The commercial inoculum used is Raprima, license from the Indonesian Institute of Sciences (LIPI). The parameters to be observed include variations in temperature and stirring time, and the concentration of metal salt. The effect of culture media, cultivation of fungi, incubation time was also investigated. UV-Vis analysis is an initial analysis to indicate the formation of nanoparticles according to the SPR peak. Then, these results were confirmed by XRD and SEM analysis. Henceforth, the nanoparticles obtained from biosynthesis can be used in certain applications.

EXPERIMENT

Chemicals and instrumentation

Chemicals used are silver nitrate (Sigma), Copper sulfate (Merck), ethanol (Merck), Potato Dextrose Broth (PDB) (Himedia), Potato Dextrose Agar (PDA)(Merck), *Rhizopus oligosporus* inoculum (Raprima) and aquadest. The tools used are a set of glassware, filter paper, hotplate, stirrer, thermometer, centrifuge, UV-Vis (Shimadzu), FTIR (Shimadzu), XRD (PanAnalytical, Type: E'xpert Pro), and SEM-EDX (FEI, Type: Inspect-S50)

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Procedure reaction

Inoculum culture of *Rhizopus oligosporus*

Culture media was prepared by weighing 1.75 grams of PDA which dissolved in 50 mL of distilled water and 1 gram of PDB which dissolved in 100 mL of distilled water. PDA and PDB solutions were sterilized using an autoclave with pressure of 15 psi, in 121ºC for 15 minutes. The PDA solution was then poured into a Petri dish and allowed to stand for 24 hours so that it hardened. After it hardens, *Rhizopus oligosporus* inoculum was sprinkled on the surface of the PDA. The fungal inoculum was incubated for 48 hours until mycelium filled the Petri dish. Furthermore, the growing fungi were taken by dividing the fungi that grow on a petri dish into 2 parts. The remains of PDA attached to the fungi was cleaned, then transferred into the PDB solution.

Biosynthesis of AgNP and Cu_2O/CuO-NP with various concentrations

Solution of 100 mL silver nitrate 0.1 M, and 50 mL PDB solution containing *Rhizopus oligosporus* were stirred for 24 hours and heated at a temperature of 40ºC. For UV-Vis analysis, samples were centrifuged for 5 minutes. The filtrate obtained was used for UV-Vis analysis. These steps were repeated using silver nitrate 0.05 M and 0.01 M. UV-Vis analysis was carried out for all variations of concentration. Then the best concentration was taken from the UV-Vis analysis results for further step.

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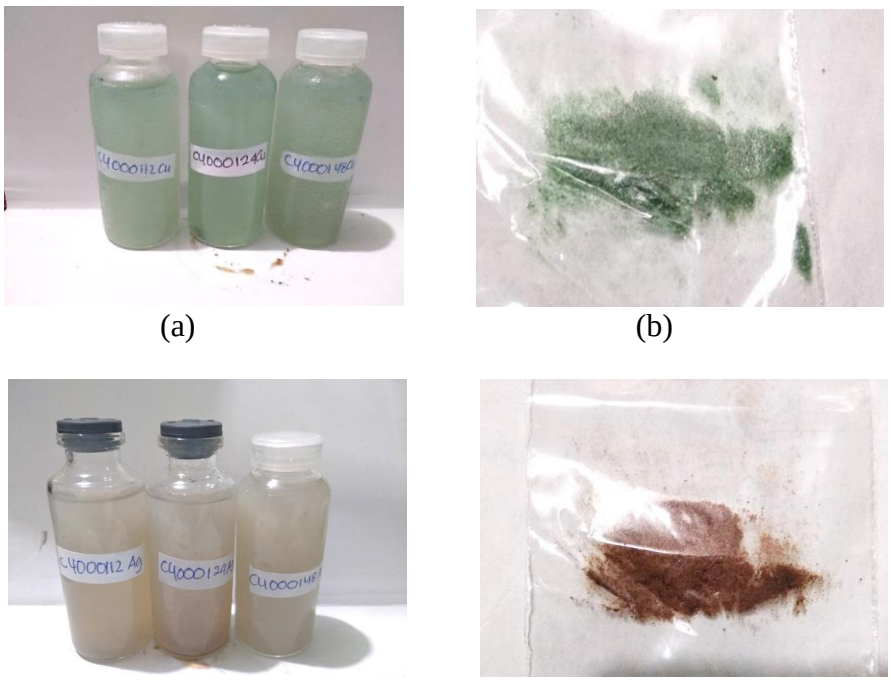
Biosynthesis of AgNP and Cu_2O/CuO-NP with various temperature and stirring time

Solution of 100 mL silver nitrate 0.1 M and 50 mL PDB containing *Rhizopus oligosporus* were stirred for 12 hours without heating. Samples were taken and the remaining solution was continued for stirring in 24 hours. Then the sample was taken and the remaining solution was continued for stirring in 48 hours. The sample was taken for further analysis. These steps were repeated for different temperature, 40ºC and 60ºC. For UV-Vis analysis, every sample were centrifuged for 5 minutes. The filtrate obtained was used for UV-Vis analysis. Furthermore, the synthesis results were taken with the best stirring time and temperature conditions to be characterized using FTIR, XRD and SEM.

RESULT AND DISCUSSION

Characterization of Metal Nanoparticles Obtained

The resulted nanoparticles were obtained from the best condition of biosynthesis and characterized using UV-Vis, FTIR, XRD and SEM. UV-Vis spectroscopy is a widely used technique to characterize the structure of nanoparticles. The resulting spectra show the surface plasmon resonance (SPR) peak which is specific for the particular metal nanoparticle. The colloidal solution of Cu and Ag are presented in figure 1a and 1c. The solution was dried and become green powder for Cu and brown powder for Ag as presented in figure 1b and 1d. The filtrate and precipitate from the centrifugation process were tested using the UV-Vis instrument. It was found that the filtrate produces the SPR peak while the precipitate does not, which is reasonable because the precipitate contains more culture media and residual fungi than metal nanoparticles.



(a) (b)

(c) (d)

Figure 1. colloidal solution (a), dried powder (b) of biosynthesized Cu in 12, 24, 48 hours, 40°C, $CuSO_4$ 0.1 M; colloidal solution (c), dried powder (d) of biosynthesized Ag in 12, 24, 48 hours, 40°C $AgNO_3$ 0.01 M.

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UV-Vis spectra of biosynthesis results are presented in Figure 1. The peak obtained at 326 nm from filtrate containing Cu, indicating an electronic transition from Cu metal. This result is similar as reported by Fuku (2018), which the alloy metals obtained may resulted electronic transition peak of metal [7]. However, there is no significant SPR peak of Cu_2O-NP and CuO-NP at 478 nm and 305 nm [8-9], respectively, thus it needs further instrument to define the nanoparticles obatined. While the analysis on filtrate containing Ag, the peak obtained at 357 nm showing the electronic transition of Ag (0) metal [10] and the SPR peak of AgNP was obtained at 413 nm.

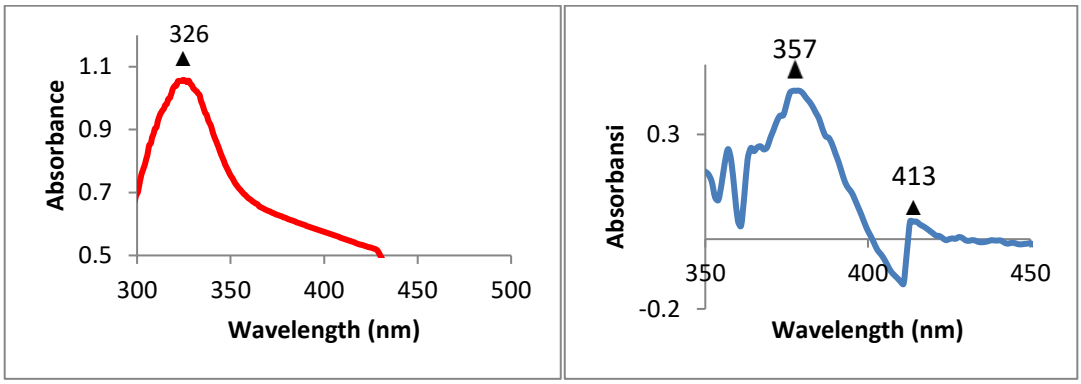

Figure 2. UV-Vis spectra of biosynthesized Cu in 12 hours, 40°C, $CuSO_4$ 0.1 M (a), UV-Vis spectra of biosynthesized AgNP in 48 hours, 40°C $AgNO_3$ 0.01 M (b)

FTIR was demonstrated to characterize the molecular structure as the UV-Vis spectra cannot define the nanoparticles contained from biosynthesis filtrate from $CuSO_4$. Figure 2 shows that there are two peaks obtained. They are peaks for the Cu (I)-O stretching vibration at 609 cm^{-1} [9,11], and Cu (II)-O bonds at 531 cm^{-1} [12]. According to this result, the particle obtained from biosynthesis is a compound which Cu binding to the oxygen.

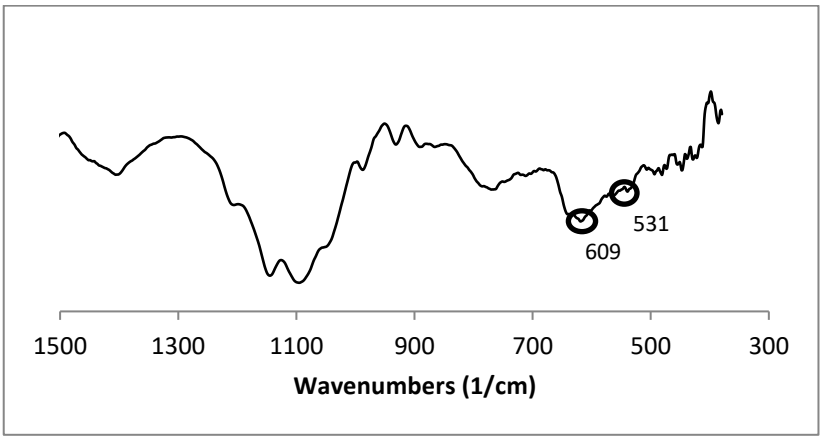

Figure 3. FTIR spectra of biosynthesized of Cu particle in 12 hours, 40°C, $CuSO_4$ 0.1 M

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To investigate the structure of synthesized particles, XRD was employed. In Figure 3, there are some peaks in which Cu_2O and CuO sharp peaks can be observed. This result is similar as reported form other research which Cu_2O and CuO have the same peak at the same 2Θ[12]. From the calculation using FWHM Data, the crystal size is 23 nm. However, the peak intensity is low because the Cu_2O and CuO particles mostly aggregate. This result is consistent with the image obtained from SEM. In magnification of 15.000, the particle obtained was not clearly visible because of the less dispersion of the particles. The chemical composition of particles obtained was determined using the EDX technique which the carbon from the culture media is the highest percentage.

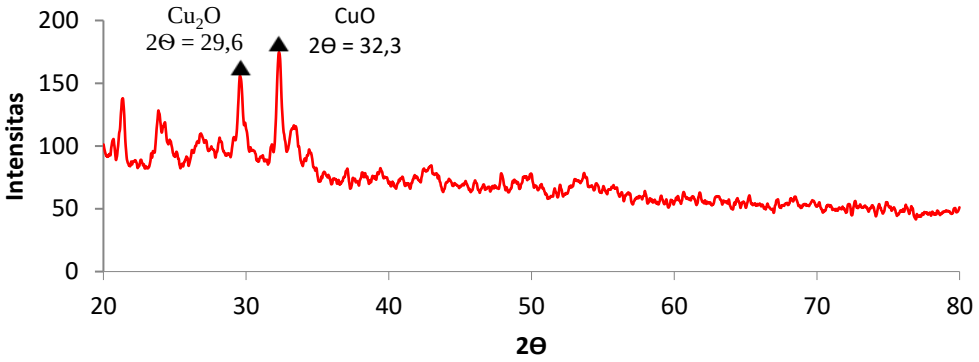

Figure 4. XRD Diffractogram of biosynthesized Cu in 48 hours, 40°C, $CuSO_4$ 0.1 M

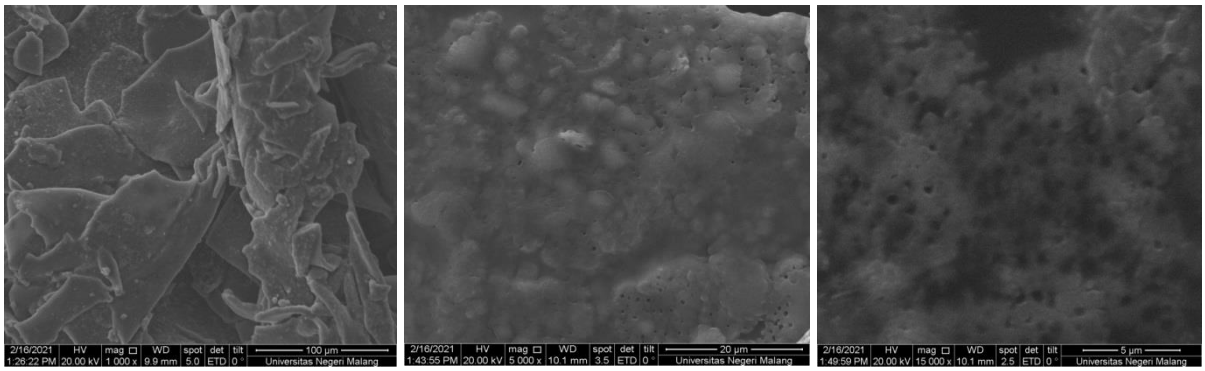

Figure 5. SEM-EDX of biosynthesized Cu in 48 hours, 40°C, from $CuSO_4$ 0.1 M in magnification of 1000x (a), 5000x (b), 15.000x (c)

From FTIR, XRD, and SEM-EDX analysis, it was confirmed that Cu_2O and CuO particles were formed although the SPR peak is not visible in UV-Vis spectra. *Rhizopus oligosporus* as the capping agent used was not effective enough to synthesis the CuNP but the reduction process of Cu^{2+} ($CuSO_4$) to Cu^{+} (Cu_2O) was quite effective.

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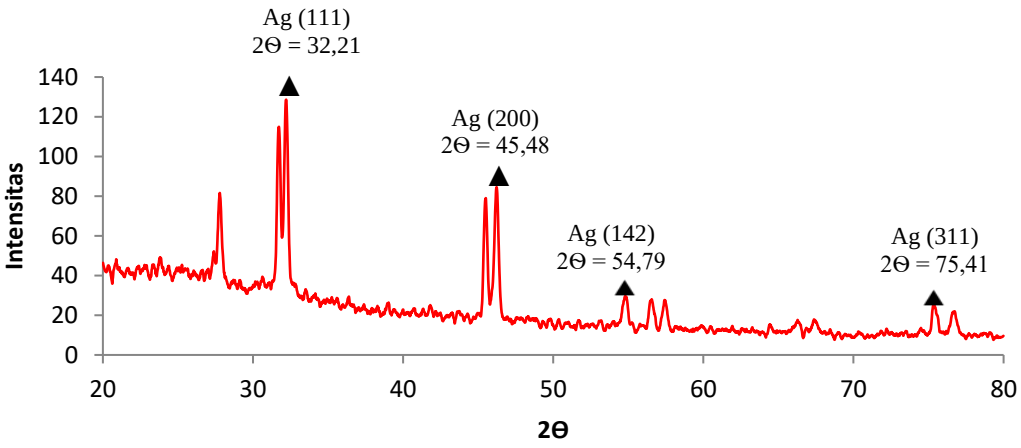

Figure 6. XRD Diffractogram of biosynthesized AgNP in 12 hours, 40°C, $AgNO_3$ 0.01 M

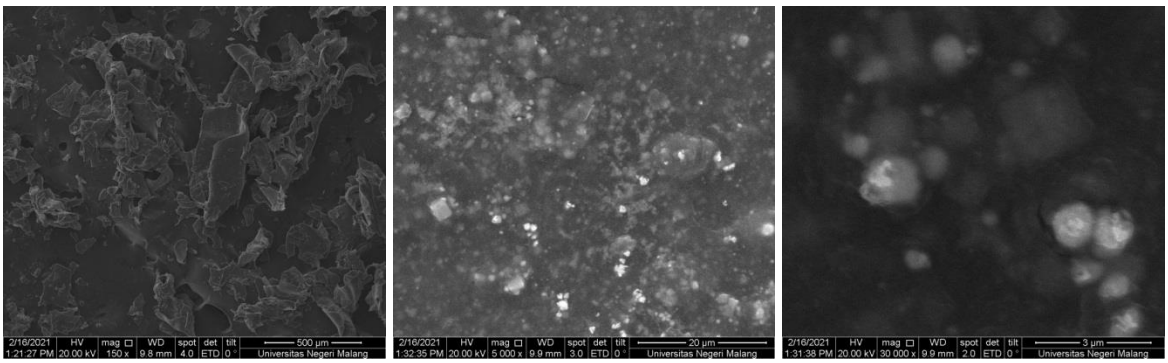

Figure 7. SEM-EDX of biosynthesized AgNP in 12 hours, 40°C from AgNP 0.01 M in magnification of 1000x (a), 5000x (b), 15.000x (c)

In figure 4, the difractogram shows some peaks corresponding to Ag which indicate (111), (200), (142), (220), and (311) [4]. However, the SEM image shows that AgNP was aggregated which is consistent to the low intensity from difractogram. However, through the FWHM data from XRD, the crystal size is 55 nm.

Analysis from instruments shows that the formation of AgNP is successful but there is some metals particle which is in phase of forming. This shows that *Rhizopus oligosporus* as reducing agent is quite effective, and the capping agent works quite well in the formation of AgNP.

The Effect of Concentration of Metal Salt, Temperature and Stirring time on Biosynthesis

Biosynthesis using fungi is related to the concentration of metal salts AgNO3 and CuSO4 used because of the influence of these metals in fungal metabolism. Fungi have a metal tolerance limit so as not to be toxic in their metabolism, so the concentration that can be reduced by fungi needs to be determined. The results of biosynthesis with various concentrations were analyzed using UV-Vis. The spectra of the solution with a certain concentration that can produce the highest absorbance is compared. It was found that the maximum tolerated and reduced metal concentrations by fungi were $CuSO_4$ 0.1 M and $AgNO_3$ 0.01 M.

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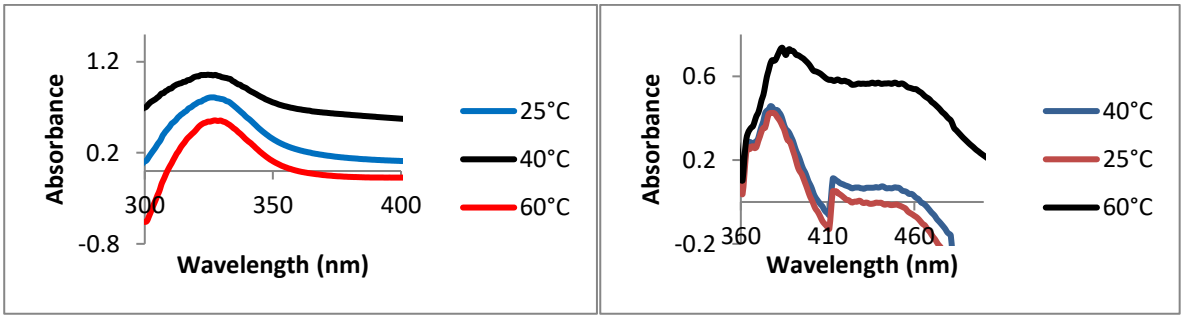
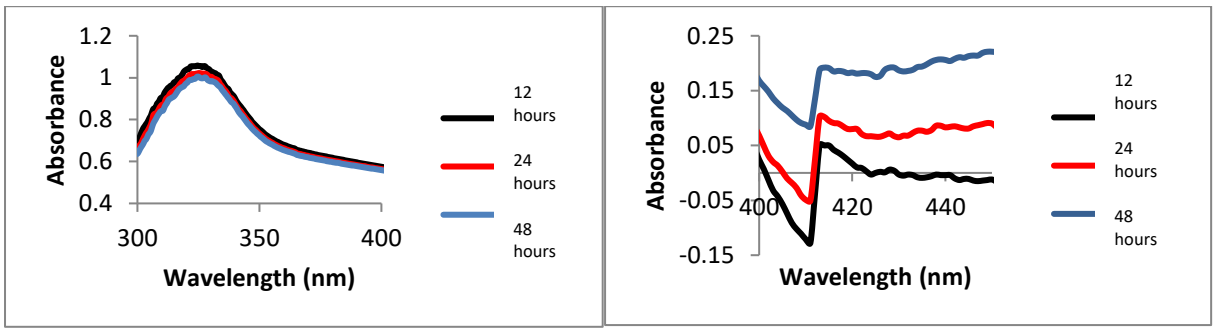
Biosynthesis using fungi is related to the enzymes contained, so that there is an optimum temperature required for the fungi to work properly. Based on the literature, fungi have an optimum temperature of 30°C - 40°C [13]. In this study, temperatures of 25°C, 40°C, and 60°C were used to determine the optimum temperature. The UV-Vis spectra results show that temperature affects the absorbance obtained. For filtrate containing Cu, there is an increasing absorbance as the temperature increases from 25°C to 40°C. However, the absorbance decreases when the temperature used is 60°C. This shows that the temperature of 40°C is the optimum temperature for the fungi to carry out biosynthesis. Meanwhile for filtrate containing Ag, the absorbance increase as the temperature increase.



(a) **(b)**

Figure 8. UV-Vis spectra of biosynthesis result to investigate the role of temperature in biosynthesis

Stirring affects the biosynthetic results due to the impact of collisions. The result of this study indicates that the absorbance increases as the stirring is longer. However, as the stirring time increase, the absorbance is not different significantly at Cu metal transition peak. Furthermore, the absorbance for Ag increase as the stirring time is longer.



(b)

Figure 9. UV-Vis spectra of biosynthesis result to compare the absorbance from biosynthesis process using culture media and cultured fungi in 40°C for 12; 24; 48 hours using 0.1 M $CuSO_4$ (a) and 0.01 M $AgNO_3$ (b).

The Effect of Culture Media on the Biosynthesis

Several studies have shown that culture media influences the size and shape of the synthesized nanoparticles. PDB culture media contain small molecules such as sugars and amino acids to polymers such as potato starch which responsible to reduce metals from its salts. In this study, biosynthesis was carried out using PDB only as a reducing agent and biosynthesis using

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fungi on PDB culture media as reducing agents. The results of the UV-Vis spectra obtained from these two processes were compared and presented in figure 5. It was found that there is a peak showing an electronic transition of Cu but there are some differences in absorbance. The results of biosynthesis using fungi on PDB culture media as reducing agents resulted in higher absorbance at the same wavelength. This shows that biosynthesis using PDB media only is not enough to reduce Cu^{2+} to Cu^{+}.

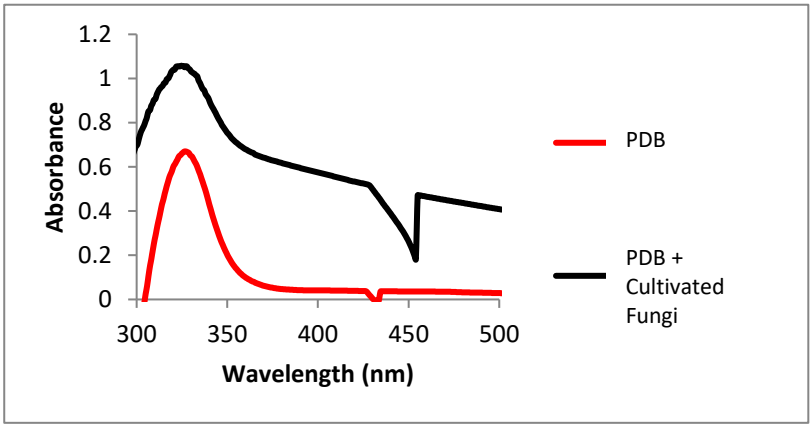

Figure 10. UV-Vis spectra of biosynthesis result to investigate the role of culture media into biosynthesis.

The Effect of Fungi Cultivation

In this study, biosynthesis was carried out using fungi in the form of inoculum and the form of culture results. This aims to determine the effectiveness of the reducing agent in cultured conditions or the form of an inoculum. The results showed that the fungi that had been cultured were more effective in reducing Cu^{2+}, wherein the biosynthetic spectra using the inoculum, no peaks showed either Cu transition or SPR CuNP peaks.

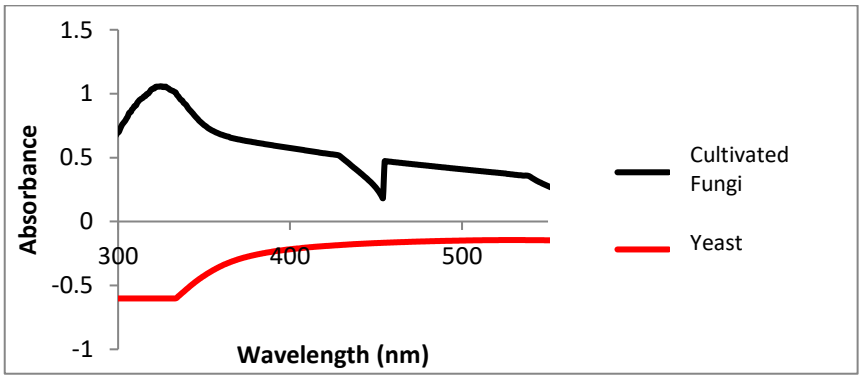

Figure 11. UV-Vis spectra of biosynthesis result to compare the effect of Fungi Cultivation

The Effect of Incubation Time on the Biosynthesis

Determining the best time for fungi harvesting is important to be used for biosynthesis. According to growth curve of fungi, there is the best phase that can produce the best biosynthesis results. In this study, 2 types of incubation out with different harvesting times were carried. The fungi were harvested on the 2nd and 5th day after cultivating in PDA and then used for

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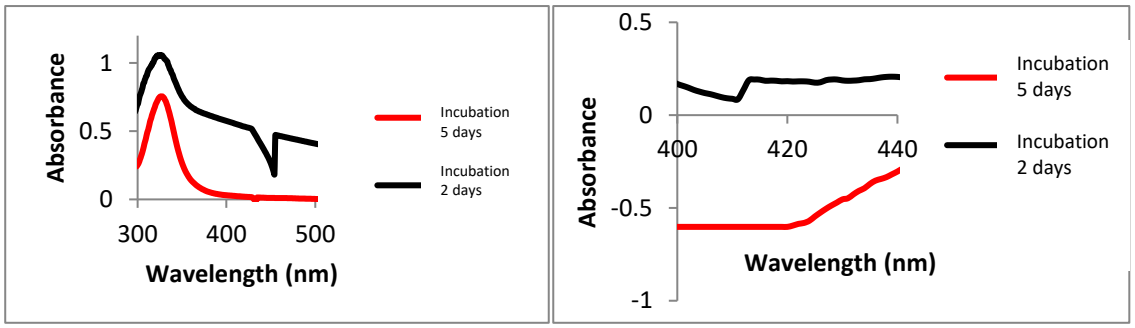
biosynthesis. The spectral results on figure 9 show that the absorbance of increases when the fungi with incubation period only for 2 days used for biosynthesis, which is reasonable because the fungi is still active in the growth phase.



(a) (b)

Figure 12. UV-Vis spectra of biosynthesis result to compare the absorbance from biosynthesis process using culture media and cultured fungi in different incubation time in 40°C for 12 hours using 0.1 M $CuSO_4$ (a) and 0.01 M $AgNO_3$ (b).

CONCLUSION

In this study, we investigated the formation of Cu_2ONP, CuONP and AgNP using *Rhizopus oligosporus* with some parameters. The parameters which investigated are concentration of metal salt, temperature and stirring time. We also investigated the effect of culture media, cultivation, and incubation time. The investigation provides the best condition to produce specific size of the nanoparticles. The best conditions are biosynthesis using *Rhizopus oligosporus* which cultivated after 2 days in PDA then transferred into PDB directly mixed with the metal salt. The biosynthesis carried out in 40°C, stirred for 48 hours. The Copper salt used is 0.1 M and silver nitrate 0.01 M. Although the particle is mostly aggregate, the grain size calculated from FWHM for Cu_2ONP, CuONP and AgNP are 23 nm and 55 nm respectively. In conclusion, this study confirms that some parameters investigated and *Rhizopus oligosporus* can be used to obtain the nanoparticles with specific size.

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