Synthesis of Cu$_2$O/biochar composites and their antibacterial activity against *Escherichia coli*

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Abstract. In this study, distillers' grains were used to prepare biochar, which is renewable and ecofriendly, then cuprous oxides nanoparticles were loaded to the biochar to improve the properties of Cu$_2$O. Their structure and morphology were characterized by X-ray diffraction (XRD), field-emission scanning electron microscope (FE-SEM), and brunauer-emmett-teller (BET) specific surface area. The results showed that the Cu$_2$O nanoparticles were loaded onto the biochar successfully and the biochar-based Cu$_2$O nanoparticles (Cu$_2$O/biochar composites) have larger specific surface area than pure Cu$_2$O nanoparticles. Their antibacterial activity against *Escherichia coli* (E. coli) was examined by tablet colony counting method and optical density (OD) method. The results showed that the Cu$_2$O/biochar composites were more effective and had superior antibacterial performance than pure Cu$_2$O nanoparticles which indicated that Cu$_2$O/biochar composites have good application prospect in antibacterial field.

1. Introduction
Nowadays, inorganic antibacterial agents, especially nanosized materials, have been paid much attention for the increasing demand for environmental sanitation [1~4]. One of the inorganic antibacterial agents is cuprous oxide and it has been used as an antibacterial agent for a long time [5]. However, Cu$_2$O particles, especially those with nanoscale structure, are easy to aggregate [6,7], which may weaken its ability in antibacterial activity [8]. Hrenovic *et al.* [9] investigated the antibacterial activity of Cu$_2$O supported onto natural clinoptilolite in the secondary effluent water and the composites showed excellent antibacterial activity. Abbasi *et al.* [10] reported the preparation of Cu$_2$O nanoparticles upon silk fibers using the ultrasound technique and they founded that the deposition of Cu$_2$O thin films on flexible polymer surfaces might be a new path for the fabrication of functional materials for different applications. Therefore, immobilizing Cu$_2$O onto support materials has been proved to be an effective strategy to improve the performance of Cu$_2$O.

Biochar refers to carbon-based dusty residues obtained from biomass pyrolysis. It has been demonstrated that biochar can reduce bioavailability of some heavy metals and that it has a high adsorption capacity to persistent organic pollutants [11]. Moreover, synthesizing biochar-based nano-composites are a new composite material and combine the advantages of biochar with nano-materials. The resulting composites usually exhibit great improvement in functional groups, pore properties, surface active sites, catalytic degradation ability and easy to separation [12]. In this study, distillers' grains were carbonized by concentrated sulfuric acid (96.5%) to get biochar. Then, a simple deposition method was applied to put Cu$_2$O onto the biochar. For this is the first time to prepare Cu$_2$O/biochar
composites, they were characterized and the antibacterial properties of them were investigated by observing their antibacterial activities to E. coli through tablet colony counting method and optical density (OD) method. As expected, the Cu$_2$O/biochar composites displayed significantly enhanced antibacterial activity.

2. Materials and Methods

2.1. Preparation of Cu$_2$O nanoparticles, biochar and Cu$_2$O/biochar composites

PEG2000, CuSO$_4$·5H$_2$O, NaOH, ascorbic acid, concentrated sulfuric acid, tryptone, yeast extract powder, NaCl, and agar were purchased from Sinopharm Chemical Reagent Co., Ltd. Distillers' grains were obtained from Fuyang (Anhui Province, China). Before experiments, distillers' grains were dried in an oven for 24 h at 105 °C and grinded into a particle.

0.1 g PEG2000 was dissolved in 20 mL of 0.5 mol/L copper sulfate solution under magnetic stirring in 250 mL round-bottom flask. Then, 40 mL of 0.5 mol/L sodium hydroxide solution was added to the above solution under magnetic stirring for 30 min. Thereafter, 50 mL of 0.1 mol/L ascorbic acid was added dropwise and stirred for 30 min. The whole reaction process was in room temperature. After that, the solution was centrifuged, washed and dried to get Cu$_2$O nanoparticles.

To prepare biochar, 20 g distillers' grains were put into 120 mL concentrated sulfuric acid under magnetic stirring for 2 h in 250 mL stand-up flask at 60 °C, then washed and dried at 80 °C in the dryer.

The synthesis of Cu$_2$O/biochar composites was similar to Cu$_2$O nanoparticles. Firstly, 2.145 g of biochar and 0.1 g PEG2000 were put into 20 mL of 0.5 mol/L copper sulfate solution under magnetic stirring in 250 mL round-bottom flask for 12 h to get more impregnation of Cu$^{2+}$ on the biochar. Secondly, 40 mL of a certain concentration of NaOH solution was added to the above solution under magnetic stirring for 30 min. Last, 50 mL of a certain concentration of ascorbic acid was added dropwise and stirred for 30 min. The whole reaction process was also at room temperature. Then, the solution was centrifuged, washed and dried at 80 °C to get Cu$_2$O/biochar composites.

2.2. Characterization Methods

The crystal structures and composition of the synthesized particles were determined by X-ray diffraction (XRD, D/MAX2500V) with a Cu Kα radiation at 40 kV and 100 mA at a scanning rate of 2°/min from 10° to 80° (20). The microstructures and morphologies of the particles were determined by field-emission scanning electron microscopy (FE-SEM, SU-8020) at 5.0 kV. The specific surface areas (SBET) of the samples were determined by an automated surface area and pore size analyzer (Autosorb-1, NOVA2200e) based on nitrogen adsorption. The effects of the particles on the growth rates of E. coli were determined by UV-visible spectrophotometer (UV-Vis, Perkin–Elmer Lambda 25).

2.3. Antibacterial Activity Experiments

Tablet colony counting method [13] was employed to investigate the antimicrobial ability of Cu$_2$O nanoparticles and Cu$_2$O/biochar composites. E. coli was selected as test strain. All the materials were sterilized in an autoclave (121 °C, 20 min) before experiments. Firstly, 1 mL E. coli was precultured in 50 mL Luria–Bertani (LB) fluid medium (5 g tryptone, 5 g NaCl, 2.5 g yeast extract powder, 500 mL H$_2$O) at 37 °C in 250 r/min oscillator for 12 h. Secondly, a certain amount of Cu$_2$O nanoparticles and Cu$_2$O/biochar composites into conical flask containing 25 mL solid LB medium (5 g tryptone, 5 g NaCl, 2.5 g extract powder, 6 g agar, 500 mL H$_2$O). Bind up and then sterilize in an autoclave (121 °C, 20 min). Thirdly, put the above solid LB medium mixed with samples into culture dish cooling to room temperature on super clean bench. Then, using SS-Spreader to spread 200μL bacterial suspension which was diluted from the above bacterial suspension to 10$^5$ CFU/mL uniformly. After that, they were incubated at 37 °C for 24 h. Antibacterial activity was evaluated by the number of formed colonies on solid LB medium.
3. Results and Discussion

3.1. Characterization of Materials

3.1.1. XRD. The XRD patterns of biochar, Cu$_2$O nanoparticles and Cu$_2$O/biochar composites are shown in Fig.1. Fig 1(a) shows a broad diffraction peak with 2θ ranged in 20°-30°, attributable to the characteristic peak of C(002). This result indicates that the biochar is composed of amorphous carbon with a low degree of graphitization [14]. The diffraction peaks of Fig.1(b) and Fig.1(c) were indexed as the (110), (111), (200), (220), (311) and (222) reflections, corresponding to the standard structure of cuprous oxide (JCPDS No. 05-0067) which indicates that Cu$_2$O nanoparticles were successfully loaded onto the biochar. What’s more, no significant peaks of impurities were detected, indicating the high purity of the obtained products. The higher (111) diffraction intensity of Cu$_2$O nanoparticles suggests that the surfaces of the Cu$_2$O nanoparticles have {111} crystal planes [15], which indicates the products are favorable for inactivating bacteria [16].

![Figure 1. X-ray diffraction patterns of: (a) biochar, (b) Cu$_2$O/biochar composites, (c) Cu$_2$O nanoparticles.](image)

3.1.2. FE-SEM. The FE-SEM images of biochar, Cu$_2$O nanoparticles and Cu$_2$O/biochar composites are shown in Fig.2. Fig.2(a) shows the FE-SEM image of biochar and reveals that it has rough surface which is in favor of loading Cu$_2$O [17]. Fig.2(b) shows the FE-SEM image of Cu$_2$O nanoparticles, the particle size of Cu$_2$O nanoparticles are close to 200 nm, as we can see from the image, it has serious aggregate problem. Fig.2(c) shows the FE-SEM image of Cu$_2$O/biochar composites. In contrast, there was no obvious aggregation of Cu$_2$O on the surface of composites, the existence of biochar make Cu$_2$O nanoparticles avoid serious aggregation and disperse homogeneously.

![Figure 2. FE-SEM images of (a) biochar, (b) Cu$_2$O nanoparticles, (c) Cu$_2$O/biochar.](image)
3.1.3. BET specific surface area. Table 1 presents the BET specific surface area (S_{BET}) data of pure Cu_{2}O nanoparticles, biochar and Cu_{2}O/biochar composites. Compared with pure Cu_{2}O nanoparticles catalyst and biochar, the S_{BET} of Cu_{2}O/biochar composites show much higher values. It may be due to that Cu_{2}O/biochar composites have less aggregate problem than pure Cu_{2}O nanoparticles as we can see from the FE-SEM.

**Table 1.** BET specific surface area of pure Cu_{2}O nanoparticles, biochar and Cu_{2}O/biochar composites.

| Material                        | pure Cu_{2}O nanoparticles | biochar       | Cu_{2}O/biochar composites |
|---------------------------------|----------------------------|---------------|-----------------------------|
| S_{BET} (m^{2}/g)               | 7.764                      | 11.607        | 24.634                      |

3.2. Comparison of Antibacterial Activity in Killing E. coli between Pure Cu_{2}O Nanoparticles and Cu_{2}O/biochar Composites

Before the comparisons of antibacterial activity in killing E. coli between pure Cu_{2}O nanoparticles and Cu_{2}O/biochar composites, a series of experiments has been down to see if the biochar has antibacterial activity. The results are negative. One of the experiments adopted tablet colony counting method. The results were shown in Fig.3. As we can see from it, the biochar has no antibacterial activity.

**Figure 3.** The comparison of antibacterial activity between biochar and pure Cu_{2}O Nps. (a: blank control, b: biochar, c: Cu_{2}O nanoparticles)

3.2.1. MIC. To quantitatively evaluate the antibacterial effects of the Cu_{2}O nanoparticles and Cu_{2}O/biochar composites, their minimal inhibitory concentrations (MIC) to E. coli were detected with agar dilution method [12]. Cu_{2}O nanoparticles and Cu_{2}O/biochar composites were dispersed into agar medium. After solidification, E. coli was inoculated on the above agar medium. Then, the growth of bacteria was observed after cultivated for 24 h in a constant temperature incubator at 37 °C. The lowest concentration, at which no visible bacterial colony could be observed, was considered to be the MIC of the antibacterial materials. The results were shown in the table 2. As we can see from table 2, the MIC of Cu_{2}O/biochar composites is lower than pure Cu_{2}O nanoparticles, which indicates that Cu_{2}O/biochar composites have better antibacterial activity than pure Cu_{2}O nanoparticles.

**Table 2.** MICs of the pure Cu_{2}O nanoparticles and Cu_{2}O/biochar composites.

| Synthetic samples | pure Cu_{2}O nanoparticles | Cu_{2}O/biochar composites |
|-------------------|----------------------------|-----------------------------|
| MIC(μg/mL)        | 56                         | 40                          |

3.2.2. The effects of particles on the growth rates of E. Coli. Prepare three sterilized 50 mL LB fluid medium, add 10 mg Cu_{2}O nanoparticles and 25 mg Cu_{2}O/biochar composites to two of them respectively, the rest as a blank control. Under UV light irradiation for 30 min in super clean workbench, then access 1mL bacteria which cultivated for 12 h to each liquid medium. The inoculated E. coli cells were grown further in a constant temperature incubator at 37 °C for various incubation times. Take out 300μL E. coli cells from them and diluted with 2700μL sterile water. Then with sterile
water as a reference, the *E. coli* densities were determined by the optical density (OD) at 600 nm. With time as the abscissa, OD value as the ordinate, draw the growth curve [14].

![Growth Curve](image)

**Figure 4.** The comparison of antibacterial activity between biochar and pure Cu$_2$O Nps. (a: blank control, b: biochar, c: Cu$_2$O nanoparticles)

Fig.4 shows that Cu$_2$O / biochar composites have apparent better antibacterial activity than pure Cu$_2$O nanoparticles. It may be due to that the cuprous oxide in composite materials has less serious aggregate problem (as we can see from the SEM) and higher specific surface area (as we can see from the table 1). The figure also shows that the biochar has no antibacterial activity. At present, many experimental results have showed that the antibacterial mechanism of cuprous oxide nanoparticles is a contact killing through which bacteria initially suffers severe damage to the cell envelope. Then further damage ensues by an independent pathway of each cuprous oxide nanoparticle [18]. Thus, a higher specific surface area contributes to the improvement of antimicrobial activity [8], which accounts for that Cu$_2$O/biochar composites have apparent better antibacterial activity.

3.2.3. Antibacterial validity. In order to explore the antibacterial validity of Cu$_2$O/biochar composites, 20 mg Cu$_2$O nanoparticles, 50 mg Cu$_2$O/biochar composites were put into 100 mL physiological saline respectively for two months. After two months, the liquid and the solid were seperated. 100 mL saline was added into the solid and marked with b, c respectively, b was solid of Cu$_2$O nanoparticles and c was solid of Cu$_2$O/biochar composites. a was blank control. After sterilization, 200μL *E. coli* which was cultivated for 12h was added to a, b and c. Put them in 250 r/min oscillator at 37 ℃ for 10 min and then coat them to the prepared solid LB medium. Antibacterial activity was evaluated after being cultivated in constant temperature incubator for 24 h. The results show in Fig.5. As we can see from the Fig.6, Cu$_2$O /biochar composites have better antibacterial activity than Cu$_2$O nanoparticles after two months. That is to say, Cu$_2$O /biochar composites have longer antibacterial validity.
Figure 5. Antibacterial validity of pure Cu2O nanoparticles and Cu2O/biochar composites. (a: blank control, b: Cu2O nanoparticles, c: Cu2O/biochar composites)

4. Conclusions
In summary, a simple deposition method was adopted to synthesize Cu2O nanoparticles. Distillers' grains and concentrated sulfuric acid were used to prepare the biochar. Then chemical precipitation was adopted to load Cu2O nanoparticles onto the biochar to improve the properties of Cu2O. The samples were characterized by XRD, FE-SEM and BET. The characterizations showed that the particle size of pure Cu2O nanoparticles is about 200nm with {111} crystal planes. Cu2O nanoparticles are loaded onto the biochar successfully and Cu2O/biochar composites have larger specific surface area and lower aggregation than pure Cu2O nanoparticles. Besides, their antibacterial activity against E. coli was compared by MIC, the growth rates of E. coli and antibacterial validity. The results showed that the Cu2O/biochar composites were more effective and had superior antibacterial performance than pure Cu2O nanoparticles. Thus, biochar is a good carrier for Cu2O nanoparticles and Cu2O/biochar composites have good application prospect in antibacterial field.

Acknowledgements
Financial support from the National Natural Science Foundation of China (No. 21277039, 21671052 and 81573399) is acknowledged.

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