

Biosynthesis, characterization and antimicrobial activity of copper oxide nanoparticles (CONPs) produced using brown alga extract (*Bifurcaria bifurcata*)

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Abstract Recently, biosynthesis of nanoparticles has attracted scientists' attention because of the necessity to develop new clean, cost-effective and efficient synthesis techniques. In particular, metal oxide nanoparticles are receiving increasing attention in a large variety of applications. However, up to now, the reports on the biopreparation and characterization of nanocrystalline copper oxide are relatively few compared to some other metal oxides. In this paper, we report for the first time the use of brown alga (*Bifurcaria bifurcata*) in the biosynthesis of copper oxide nanoparticles of dimensions 5–45 nm. The synthesized nanomaterial is characterized by UV–visible absorption spectroscopy and Fourier transform infrared spectrum analysis. X-ray diffraction confirms the formation and the crystalline nature of copper oxide nanomaterial. Further, these nanoparticles were found to exhibit high antibacterial activity against two different strains of bacteria

Enterobacter aerogenes (Gram negative) and *Staphylococcus aureus* (Gram positive).

Keywords Green synthesis · Copper oxide nanoparticles · Brown alga · Antimicrobial activity

Introduction

Unlike bulk materials, nanoparticles have been intensively studied over the last decade due to their characteristics: physical, chemical, electronic, electrical, mechanical, magnetic, thermal, dielectric, optical and biological properties (Schmid 1992; Daniel and Astruc 2004). Therefore, nanoparticles are considered as building blocks of the next generation of technology with applications in many industrial sectors. In particular, metal oxide nanoparticles are receiving increasing attention in a large variety of applications. Metal oxide nanoparticles are of interest because of their unique optical, electronic and magnetic properties. The oxides of transition metals are an important class of semiconductors, which have applications in magnetic storage media, solar energy transformation, electronics, gas sensors and catalysis (Ramgir et al. 2013; Jani et al. 2013; Shalana et al. 2013; Montferrand et al. 2013; Ahmadi et al. 2011). Although various physical and chemical methods have been extensively used to produce nanocrystalline copper oxide such as microemulsion method (Nassar and Husein 2007), arc-submerged nanoparticle synthesis system (Kao et al. 2007), flame-based aerosol methods (Chiang et al. 2012), sonochemical (Vijayakumar et al. 2001), hydrothermal (Zhang et al. 2006) and solid-state techniques (Wang et al. 2004), the stability and the use of toxic chemicals are subjects of paramount concern. The use of toxic chemicals on the

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surface of nanoparticles and non-polar solvents in the synthesis procedure limits their applications in clinical fields. Therefore, development of clean, biocompatible, nontoxic and eco-friendly methods for nanoparticles synthesis deserves merit. The interest in this field has shifted toward 'green' chemistry and bioprocess approach. These approaches focus on utilization of environment-friendly, cost-effective and biocompatible reducing agents for synthesis of copper oxide nanoparticles (CONPs). Review of literature revealed that synthesis of CONPs using microorganisms and plant extract has been unexplored; there are only a very few reports on the use of yeast, fungi, bacteria or plant extract for synthesizing CONPs (Honary et al. 2012; Rahman et al. 2009; Gunalan et al. 2012) compared to the great number of articles for other metals, and none using the brown alga (*Bifurcaria bifurcata*). Therefore, in the present work, attempts were made to utilize the potential of this brown alga as a biofactory for the CONPs synthesis. Interestingly, this is the first report on the synthesis of highly stable CONPs using marine alga (*Bifurcaria bifurcata*). The process is described and different analytical techniques were used including UV–visible spectroscopy (UV–vis), Fourier transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM) and X-ray diffraction analysis (XRD). Furthermore, the bacterial effect of CONPs was also analyzed by disc diffusion method.

Materials and methods

Preparation of alga extract

Bifurcaria bifurcata, a brown alga, collected on rocks at about 3 m depth from Morocco's Atlantic Coast in Rose Marie near Rabat, was extensively washed with deionized water, until the pH of the wash solution was equal to deionized water, and subsequently dried in an oven at 60 °C overnight. It is then finely powdered and stored. About 10 g of powdered alga was transferred into a 150 ml beaker containing 50 ml double distilled water, mixed well on rotary shaker for 1 h and then boiled for 15 min. The extract obtained was filtered and used as a reducing agent and stabilizer.

Synthesis of copper oxide nanoparticles

Synthesis of colloidal CONPs has been done using the following procedures; typically 2 ml of alga extract was added dropwise into 20 ml of 1 mM aqueous solution of copper(II) sulfate with constant stirring at 100–120 °C. Within few hours, the deep blue solution gradually became colorless and then turned slowly to brick red coloration which changed to dark after vigorous stirring for 24 h.

Characterization of copper oxide nanoparticles

The bioreduction of copper (II) ion in solution was monitored using Perkin-Elmer Lambda 2 double beam UV–visible spectrometer against distilled water as blank. After that, the solution mixture (alga extract and copper sulfate) was centrifuged at 5,500 rpm for 15 min and subsequently redispersed in distilled water to get rid of any uncoordinated biological molecules. This process of centrifugation was repeated thrice to ensure better separation of the CONPs. The purified dried powders were then used for further characterization. The FTIR spectral measurements were carried out on Bruker Tensor-27 spectrophotometer and the XRD analysis was obtained using Bruker D8 Advance powder X-ray diffractometer. The morphology of the nanoparticles was analyzed using the high-resolution image obtained with Tecnai G2 transmission electron microscope.

Antibacterial activity of synthesized copper nanoparticles

The CONPs synthesized using alga extract were tested for antimicrobial activity by agar disc diffusion method against pathogenic bacteria *Enterobacter aerogenes* (Gram-negative) and *Staphylococcus aureus* (Gram-positive). The pure cultures of bacteria were subcultured on nutrient agar medium. Each strain was swabbed uniformly onto the individual plates using sterile cotton swabs. Filter paper discs (Whatman no. 3, 6 mm diameter) were sterilized by autoclaving. 20 µl of nanoparticle solution was loaded onto each paper disc and allowed to air dry. The dry discs were placed on the previously inoculated agar. After incubation at 37 °C for 24 h, the different levels of zone of inhibition of bacteria were measured.

Results and discussion

The formation of CONPs was initially confirmed visually and by using UV–visible spectroscopy technique which has been frequently used to characterize the synthesized metal and metal oxide nanoparticles. The change in color of the reaction mixture (Fig. 1) due to surface plasmon resonance phenomenon provides a convenient signature to indicate the formation of CONPs in the reaction mixture (Krithiga et al. 2013).

Figure 2 shows the UV–vis absorption spectra recorded for alga extract before (curve A) and after (curve B) the reaction with 1 mM copper(II) sulfate. As seen, the UV–visible absorption spectrum of the solution containing alga extract before addition of copper sulfate used as a control (curve A) shows two peaks of maximum absorption at 245



Fig. 1 Color change during the phyto-reduction of CuSO_4 into CONPs at the beginning and after 48 h of reaction

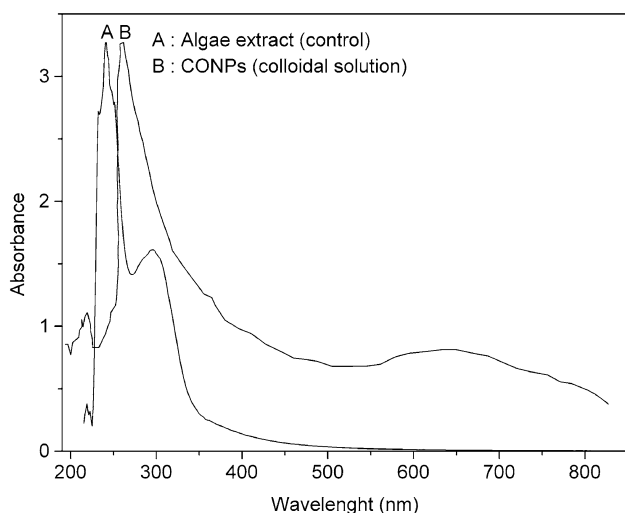


Fig. 2 UV–vis absorption spectrum of CONPs prepared with alga extract. *A* Alga extract as control; *B* solution formed at the end of the reaction

and 290 nm characteristic of diterpenoids that are very abundant in the brown algae *Bifurcaria bifurcata* (Valls et al. 1993; Combaut and Piovetti 1983). After addition of copper sulfate (curve B), it is clearly seen that these bands disappeared and new bands were observed in the solution. The colloidal suspensions after reduction (curve B) show two strong resonance, one at about 260 nm which are attributed to the formation of cuprous oxide nanoparticles (Cu_2O) (Borgohain et al. 2002) and another weak broad resonance centered at about 650 nm, which is assigned to the formation of cupric oxide nanoparticles (CuO) (Yin et al. 2005). The observed changes in the spectrum reflect the characteristic pattern of CONPs formation by reducing copper ions with water-soluble diterpenoids present in the algae extract. These results clearly suggest the formation of a mixed Cu_2O and CuO phase in colloidal solution that is in conformity with available literature. It is important to note that lot of reports in the literature on synthesis of copper/CONPs by various techniques have commonly resulted in a mixture of CuO and Cu_2O nanocrystals

[Yin et al. 2005; Rahman et al. 2009; Swarnkar et al. 2009]. The shape and size of the resultant particles were elucidated with the help of TEM (Fig. 3). Nanoparticles observed from the micrograph majority are spherical with a small percentage of elongated particles. It is observed that there is a variation in particle size. Most of the nanoparticles ranged from 5 to 45 nm in size, and the average size estimated was 20.66 nm according to the size distribution shown in (Fig. 4). Further, evidence for the biosynthesis of CONPs is provided by the XRD pattern of the synthesized nanopowder illustrated in Fig. 5. It can be seen that the as-prepared sample clearly indicates the presence of two crystalline phase indices, monoclinic cupric oxide (CuO)

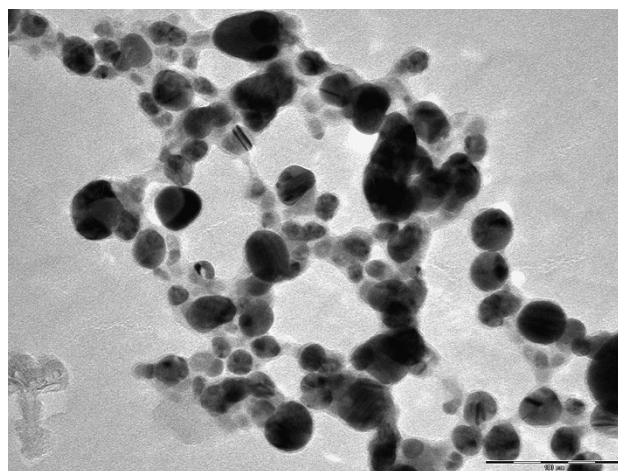


Fig. 3 TEM image of CONPs synthesized from the alga extract

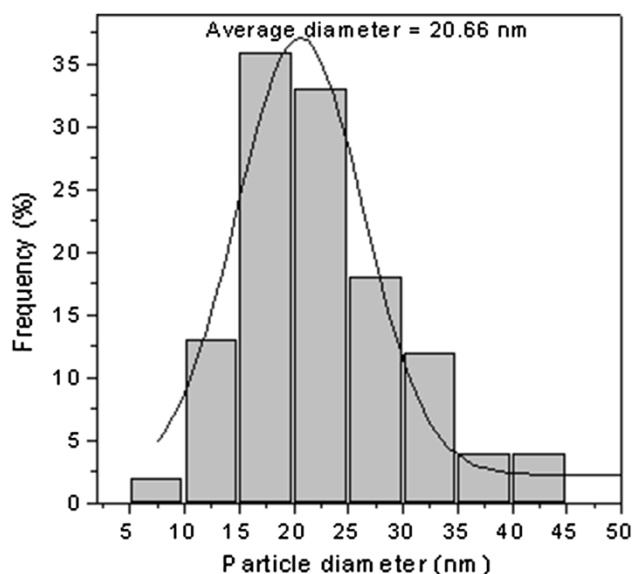


Fig. 4 Histogram of the particle size distribution of CONPs synthesized from the alga extract

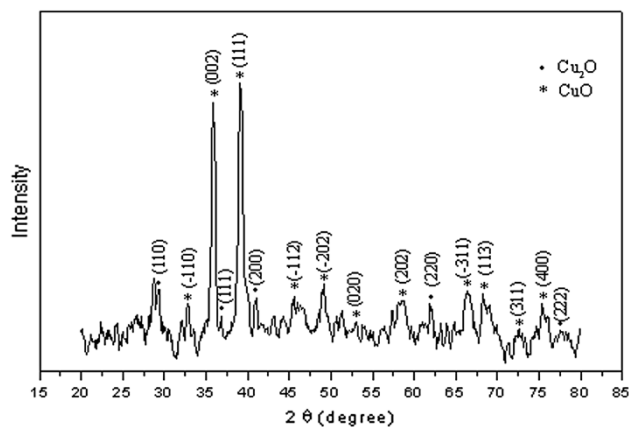


Fig. 5 XRD spectrum of CONPs synthesized from the alga extract

and cubic cuprous oxides (Cu_2O). The peak positions with 2θ values of 29.4° , 36.8° , 42.1° , 61.9° and 77.6° are indexed as (110), (111), (200), (220) and (222) planes which are in good agreement with those of powder Cu_2O obtained from the International Center of Diffraction Data card (JCPDS file no. 05-0667) confirming the formation of a crystalline cubic phase Cu_2O with a cuprite structure (Kooti and Matouri 2010; Srivata et al. 2013), while the peaks at 32.8° , 35.9° , 39.1° , 46.3° , 49.1° , 52.9° , 58.7° , 66.6° , 68.3° , 72.6° and 75.5° can be assigned to (-111) , (002), (111), (-112) , (-202) , (020), (202), (-311) , (113), (311) and (400) planes which are matched with the values of monoclinic phase CuO reported in the literature (Volanti et al. 2008) and with the respective “JCPDS” (Joint Committee on Powder Diffraction Standards card no. 45-0937). Therefore, the formation of both Cu_2O and CuO nanocrystals in the sample was confirmed by the above XRD studies. This result is in agreement with that of UV–visible absorption spectroscopy. Higher intensity at 2θ values of 35.9° and 39.1° respectively indicates that the mixed phase has major proportion of CuO with the highly oriented crystalline CuO phase. Thus, the amount of cuprous oxide is less than that of cupric oxide. The average particle size was estimated using the well-known Scherrer formula, $D = k\lambda/\beta\cos\theta$, where D is particle diameter, k is a constant equals 1, λ is wavelength of X-ray source (0.1541 nm), β is the full width at half maximum (FWHM) and θ is the half diffraction angle. The particles sizes obtained from XRD line broadening was found to be around 18.34 nm which well correlates with that obtained from TEM. Thus the XRD pattern proves to be strong evidence in favor of the UV–vis spectra and TEM images for the presence and size of copper oxide nanoparticles.

FTIR measurements of both the aqueous alga extract and the synthesized dried CONPs were carried out to identify the possible biomolecules responsible for the reduction, capping of and efficient stabilization of the bio-

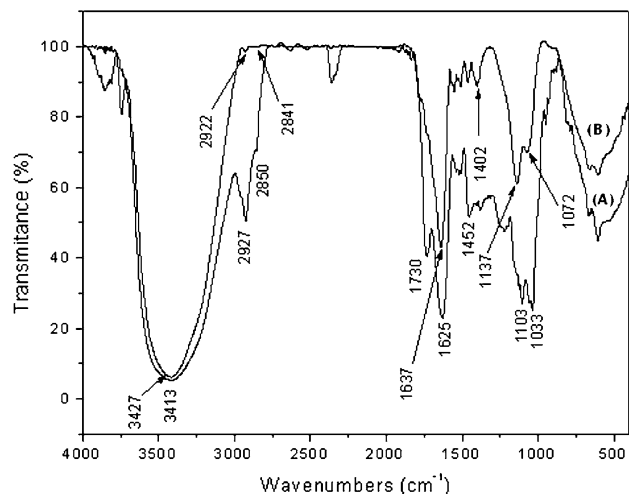


Fig. 6 FTIR spectra A alga extract; B CONPs nanoparticles

reduced CONPs. The FTIR spectra of the alga extract and the synthesized CONPs are shown in Fig. 6a and b. The alga extract displays a number of adsorption peaks, reflecting its complex nature. A wider range of compounds has been reported from the brown alga of the genus *Bifurcaria bifurcata*, when diterpenoids were the predominantly reported compound classes [Blunt et al. 2005; Amico 1995]. As shown in Fig. 6a, diterpenoids from *Bifurcaria bifurcata* can be identified by the strongest peaks of hydroxyl at $3,413\text{ cm}^{-1}$, α , β -unsaturated ketone band at $1,730\text{ cm}^{-1}$, olefinic band at $1,625\text{ cm}^{-1}$, primary and secondary alcohols functionalities bands at $1,103$ and $1,033\text{ cm}^{-1}$ as well as the peaks around $3,000$ and $1,400\text{ cm}^{-1}$ attributable to aliphatic C–H stretching and bending modes [Magné et al. 2005; Culioli et al. 1999]. By comparing the spectrum of CONPs with that of the alga extract, we can conclude that the two spectra are similar in their spectral features. There is no question, therefore, that the compound on the surface of CONPs has a very close chemical composition to the alga extract if not identical. It was found that many peaks obtained by the alga extract have been repeated in the FTIR spectrum of CONPs with changes in the position as well as in the intensity of absorption bonds. After the synthesis of CONPs, the absorption peaks at $3,413$, $1,625$, $1,103$ and $1,033\text{ cm}^{-1}$ corresponding to OH, C=C and C–O observed in plant extract get narrower and shifted to higher frequency regions, while those at around $3,000$ and $1,400\text{ cm}^{-1}$ attributable to aliphatic C–H stretching and bending modes decreased in intensity and shifted to low frequency regions. In addition the disappearances of $\nu_{\text{C=O}}$ stretching vibration of the α , β -unsaturated ketone at $1,730\text{ cm}^{-1}$ confirm that the reduction and the stabilization of CONPs proceed via these groups which confirm that water-soluble compounds such as diterpenoids are present in *Bifurcaria Bifurcata*

extract has the ability to perform dual functions of reduction and stabilization of CONPs. A similar observation has been reported by several works [Shankar et al. 2004; Rajathi et al. 2012; Kiruba Daniel et al. 2012).

The antibacterial activity of alga extract and CONPs was examined against both Gram-negative and Gram-positive bacteria by using disc diffusion test. The radial diameter of the inhibition zone of *E. aerogenes* and *S. aureus* by CONPs are 14 and 16 mm, respectively (Fig. 7). The discs filled with alga extract alone did not show any zone of inhibition suggesting that at the concentration used for synthesis of CONPs, alga extract showed no antibacterial activity, but CONPs synthesized from the extract exhibited good antibacterial activity. Based on these results, it can be concluded that these synthesized CONPs had significant antibacterial action on both of the Gram classes of bacteria, which may be attributed to the greater abundance of amines and carboxyl groups on their cell surface and greater affinity of copper ions toward these groups (Beveridge and Murray 1980). CONPs show efficient antibacterial property due to their extremely large surface area, which provides better contact with microorganisms. Copper ions released subsequently may bind with DNA molecules and lead to disorder of the helical structure by cross-linking within and between the nucleic acid strands. Copper ions inside bacterial cells also disrupt biochemical processes [Kim et al. 2000; Stohs and Bagchi 1995). The exact mechanism behind is not known and needs to be studied further. Furthermore, the Gram-negative bacteria seemed to be more resistant to CONPs than Gram-positive bacteria. This observation is in agreement with earlier studies (Zarindokht and Chehrizi 2011). It was earlier reported that the interaction between Gram-positive bacteria and nanoparticles was stronger than that of Gram-negative bacteria because of the difference in cell walls between Gram-positive and Gram-negative bacteria. The cell wall of *E. aerogenes*, which consists of lipids, proteins and

lipopolysaccharides (LPS), provides effective protection against biocides. However, the cell wall of Gram-positive bacteria, such as *S. aureus*, does not consist of LPS (Speranza et al. 2004).

Conclusion

In the present work, we first report an eco-friendly and convenient method for the synthesis of CONPs using brown alga (*Bifurcaria bifurcata*) extract. No chemical reagent or surfactant template was required in this method, which consequently enables the bioprocess with the advantage of being environmental friendly. The developed nanoparticles were characterized by UV–vis, TEM, XRD and FTIR measurements and showed good antibacterial activity. An important potential benefit of the described method of synthesis of nanoparticles using marine algae is that they are quite stable in solution and this is a very important advantage over other biological methods currently in use. This biosynthesis technique can be a promising method for the preparation of other metals and metal oxide nanoparticles and can be valuable in environmental, biotechnological, pharmaceutical and medical applications.

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Fig. 7 Antibacterial assay: zone of inhibition against *Enterobacter aerogenes* and *Staphylococcus aureus*

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