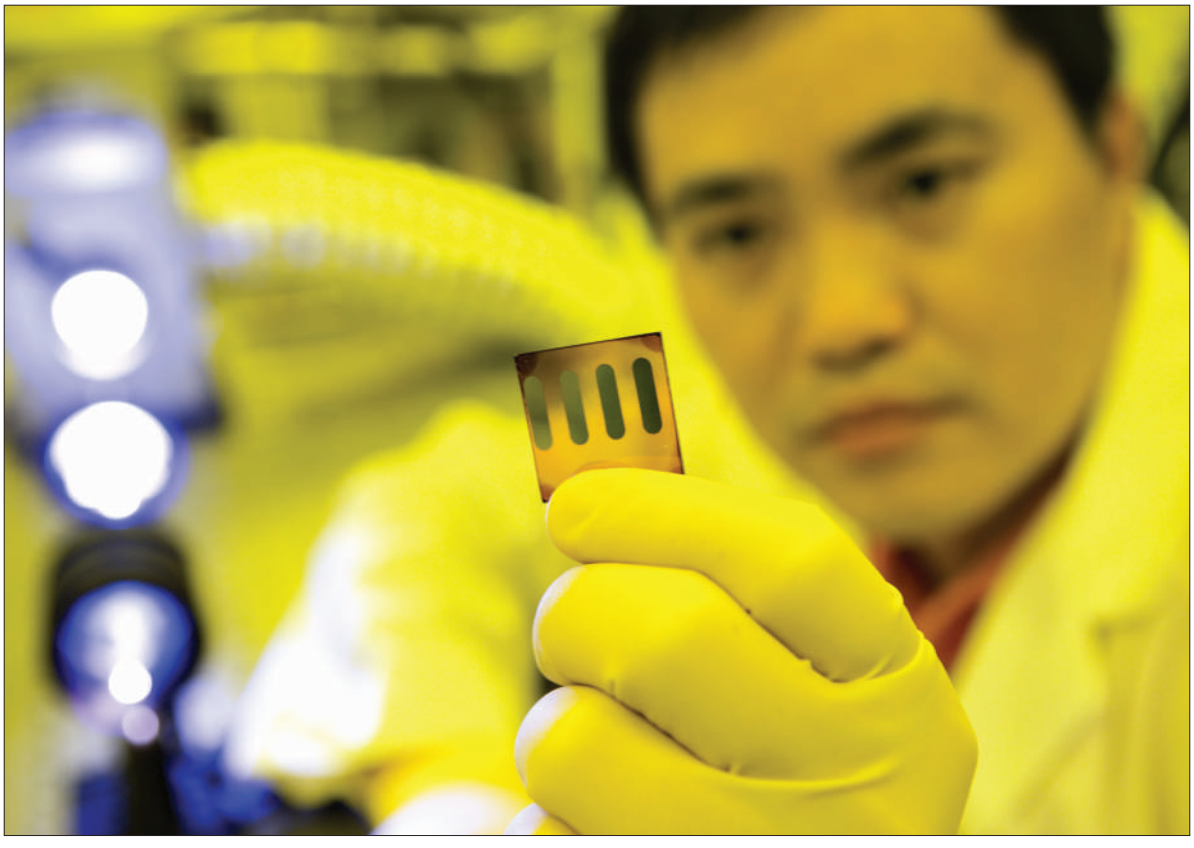

Singapore Journal of

Scientific Research

ISSN: 2010-006x

science alert

http://scialert.net/sjsr

OPEN ACCESS Singapore Journal of Scientific Research

ISSN 2010-006x
DOI: 10.3923/sjsres.2019.13.19

Research Article
Streptomyces Mediated Synthesis of Copper Oxide Nanoparticles (CuO-NPs) and its Activity Against *Malassezia furfur*

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Abstract

Background and Objective: The use of hazardous chemicals during the synthesis of nanoparticles limits their use in clinical fields. Control of *Malassezia* sp. using green synthesized nanoparticles could be an alternate treatment to avoid hazardous chemicals. This study aimed to biosynthesis and characterization of copper oxide nanoparticles using actinomycetes isolated from rhizosphere soil. Also, evaluating the efficiency of biosynthesized nanoparticles under *in vitro* controlling of dandruff causative agent was performed. **Material and Methods:** Characterization of synthesized nanoparticles (CuO-NPs) by both UV-Vis spectrophotometer and scanning electron microscope revealed the particles were of 91 nm in size. Various concentrations of CuO-NPs were tested for their efficacy against *M. furfur* MTCC 1374 and minimum inhibitory concentration values were determined through *in vitro* experiments. **Results:** Concentrations of the actinomycetes mediated CuO-NPs higher than 75 μg mL^{-1} inhibit the growth of the *Malassezia* significantly ($p<0.05$). This study proposes green-synthesis method for the production of CuO-NPs using actinomycetes with high anti-malassezial activity. **Conclusion:** Thus, this nanomaterial could be useful for controlling *Malassezia furfur* that affects humans globally.

Key words: *Streptomyces*, *Malassezia*, copper oxide nanoparticles, antidandruff, actinomycetes, rhizosphere, dandruff

Citation: Saroj Yadav, V. Latha and G. Sibi, 2019. *Streptomyces* mediated synthesis of copper oxide nanoparticles (CuO-NPs) and its activity against *Malassezia furfur*. J. Sci. Res., 9: 13-19.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Copper nanoparticles (Cu-NPs) are synthesized by hydrothermal, microwave, photochemical, electrochemical, microemulsion and chemical reduction methods[1,2]. The use of hazardous chemicals during the synthesis of nanoparticles limits their use in medical and clinical fields[3]. The high cost involved in the physical and chemical methods also contributes to the search of alternative methods to synthesize nanoparticles[4]. Synthesis of nanoparticles using micro organisms, enzymes and plant extracts has been suggested as possible biological methods to synthesize nanoparticles[5]. Biosynthesis of nanoparticles is done using a diverse group of microorganisms[6-9].

Actinomycetes are a diverse group of Gram-positive bacteria common in soil and widely distributed in various environments. Actinomycetes are widely recognized because of their potential to produce many kinds of novel secondary metabolites[10]. Antimicrobial compounds from actinomycetes were active against bacteria, fungi and viruses[10-13]. Actinomycetes have received a considerable attention as an efficient candidate for the synthesis of metal nanoparticles[9]. Actinomycetes exhibit good stability and polydispersity making them an efficient candidate for the synthesis of metal nanoparticles[14,15]. They are capable of synthesizing intracellular and extracellular nanoparticles. Electrostatic binding of Ag^+ ions to carboxylate group of mycelial cell wall enzymes leads to intracellular synthesis of nanoparticles[16].

Among the actinomycetes genera, *Streptomyces* species are regarded as a dominant contender for the biosynthesis of nanoparticles[17] and are reported to produce silver, manganese and zinc nanoparticles[18].

Copper nanoparticles have important applications in diverse fields[19-23]. CuO-NPs possess significant antimicrobial properties by inhibiting the growth of bacteria, fungi, viruses and algae[24,25]. Copper oxide in nano-size scale has longer shelf life as compared to other organic antimicrobial agents such as silver and gold[26]. Actinomycetes mediated synthesis of copper oxide nanoparticles have been reported to exhibit antibacterial properties[27,28]. The present study aimed to biosynthesis and characterization of copper oxide nanoparticles using actinomycetes isolated from rhizosphere soil. Also, evaluating the efficiency of biosynthesized nanoparticles under *in vitro* controlling of dandruff causative agent was performed.

MATERIALS AND METHODS

Study area: This study was carried out at Department of Biotechnology, Indian Academy Degree College-Autonomous, Bangalore between June, 2019 and November, 2019.

Sample collection and isolation of actinomycetes: Actinomycetes strains were isolated by means of the dilution agar plating method from rhizosphere soil. Soil samples were diluted up to 10^{-5} and aliquots (0.1 mL) were spread over the agar plates of starch casein nitrate agar medium supplemented with cycloheximide (50 µg mL^{-1}). Cultures were incubated at 28°C for 2-4 weeks. The morphological features of spores, sporangia and aerial substrate mycelium were observed and recorded. *Streptomyces* was recognized on the basis of these morphological criteria.

Preparation of cell free supernatant: The actinomycete isolate was inoculated in 250 mL flask containing Sabouraud Dextrose Agar (Dextrose 40 g L^{-1}, mycological peptone 10 g L^{-1}, agar 15 g L^{-1}, final pH (at 25°C) 5.6±0.2). The flasks were kept in the rotary shaker and incubated at 28°C for 7 days. After incubation period, the cell free supernatant was collected by centrifugation at 10,000 rpm and the supernatant obtained was used for the synthesis of nanoparticles.

Synthesis of copper oxide nanoparticles: About 25 mL of the cell free supernatant was mixed with aqueous solution of 10 mM copper sulphate [$CuSO_4.5H_2O$] and was heated at 100°C for 15 min, resulting in the color change from blue to reddish brown. Followed by centrifugation at 10,000 rpm for 15 min, the copper oxide nanoparticles (CuO-NPs) were obtained. The pellet was washed repeatedly with distilled water and centrifuged again for 10 min, then followed by ethanol wash to remove the water soluble molecules from the CuO-NPs suspension. The copper oxide nanoparticles were dried at room temperature for 48 h and stored in an airtight container until further analysis.

Characterization of copper oxide nanoparticles: The confirmation of biosynthesized CuO-NPs was carried out using a UV-VIS spectrophotometer (Systronics 117 UV-VIS spectrophotometer) at a wavelength ranging between 200-800 nm.

To study the morphology and size of CuO-NPs, scanning electron microscope (SEM) (Hitachi S 3500) was used for investigation. For this purpose, the sample was prepared by

dispensing a drop of colloidal copper solution on a carbon-coated 200 mesh copper grid and allowed to dry at room temperature (27°C) before the examination.

Test organism: *Malassezia furfur* MTCC 1374 was obtained from microbial type culture collection (MTCC), Chandigarh and was used as reference strain in this study.

Preparation of yeast inoculum: Yeast inoculums were prepared from cultured *Malassezia furfur* (MTCC 1374) on Dixon agar medium incubated at 37°C for 48 h. The yeast were transferred to Dixon broth media and incubated at 37°C overnight. One mL from each overnight broth culture was inoculated to 10 mL broth medium and incubated at 37°C on a rotary shaker with 180 rpm shaking until optical density adjusted to 0.5 McFarland standards for antifungal assay of CuO-NPs.

Antidandruff activity of actinomycetes mediated copper oxide nanoparticles: The biosynthesized copper oxide nanoparticles were tested for their antagonistic activity against *Malassezia furfur* MTCC 1374 using the well diffusion method. Twenty microlitres of CuO-NPs at different concentrations (100, 200, 300, 400 and 500 µg mL^{-1}) were pipetted into each well used. Ketoconazole was used as positive control. After incubation at 37°C overnight, the radius of the inhibition zone around each well was measured in mm.

MIC of nano-copper oxide nanoparticles: The minimum inhibitory concentrations (MICs) of CuO-NPs for *M. furfur* were determined in 96-well microtiter plate technique as described earlier by Padil and Cernik[29]. Dixon broth (DB) medium was used for resistance experiments. MIC values were detected by various concentrations of CuO-NPs in the range of 100-500 µg mL^{-1}. As the first step, 50 µL aliquot of 0.5 McFarland microbial suspension was inoculated to the wells of microtiter plate, then, 50 µL of DB supplemented with the considered concentrations of CuO-NPs was added. The DB medium without CuO-NPs inoculated with the yeast and DB medium supplemented with CuO-NPs without any yeast were used as proper controls for these experiments. After incubation at 37°C for 48 h, the micro titer plates were scanned with an ELISA reader (Genetix- 106470GB) at 600 nm. All experiments were carried out in triplicates with proper blank.

Statistical analysis: All fungal growth assays were carried out in triplicate and the data are presented as the mean standard deviation. One-way analysis of variance (ANOVA) was performed to compare the treated group and the controls (without CuO-NPs). Statistical differences were considered significant at $p<0.05$.

RESULTS

Morphological characteristics of the *Streptomyces* isolate during the study is presented in Fig.1 and Table 1.

UV-Vis spectral analysis of CuO-NPs synthesized from actinomycetes: The formation of CuO-NPs was primarily detected by visualizing the change in color of the culture filtrate to reddish brown. The UV-VIS spectra of the CuO-NPs exhibit the maximum absorption peaks at 290-300 nm suggesting CuO-NPs formation. To ascertain the formation and stability of the copper colloidal solution, UV spectroscopy is done that is associated with surface plasmon resonance (SPR) band arises due to collective oscillation of 6 s electron in the conduction band of CuO-NPs. The absorption spectrum for CuO-NPs was observed between 300 and 464 nm ranges with

Table 1: Morphological characteristics of the actinomycetes isolate

Parameters	Observations
Colony color	Grey
Colony morphology	Powdery, raised
Melanin pigment	-
Shape of spores	Oblong
Spore surface	Smooth
Color of aerial mycelium	Greyish white
Color of substrate mycelium	Pale white
Temperature	27°C (Mesophilic)

Fig. 1: *Streptomyces* isolate from the study

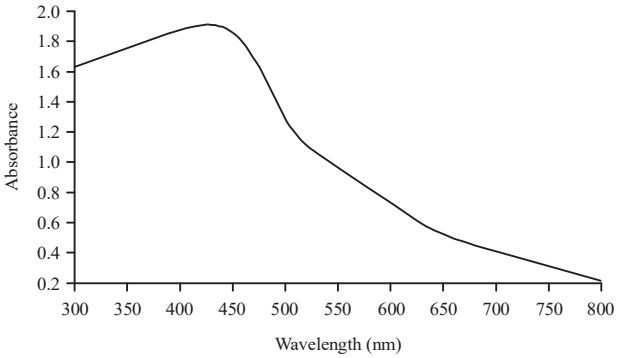

Fig. 2: UV-Vis spectrum of CuO-NPs

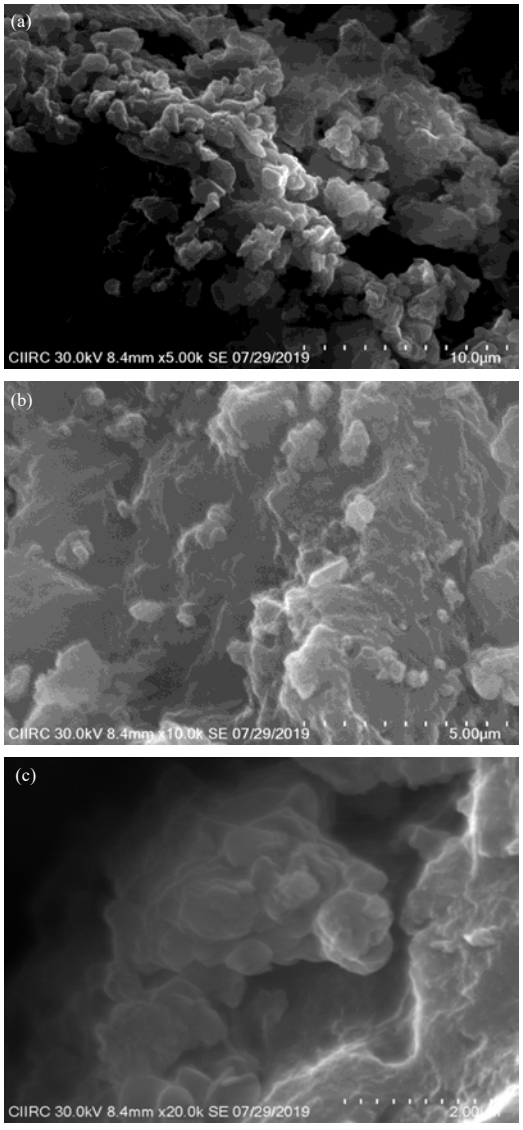

Fig.3(a-c): SEM micrographs of CuO-NPs synthesized using *Streptomyces* (a) 10 µm, (b) 5 µm and (c) 2 µm scale bar

Fig. 4: Antimalassezial activity of *Streptomyces* mediated CuO-NPs

an intense peak around 422 nm. No other measurable peak was observed in the spectrum which confirms that the synthesized products are Cu only (Fig. 2).

Scanning electron microscopy (SEM) has been employed to characterize the size, shape and morphology of synthesized copper nanoparticles. SEM images of copper nanoparticles are shown in Fig. 3a-c. The average particle size of the CuO-NPs was found to be around 91 nm. The sample studied reveal monodispersity of the metal particles.

Anti-malassezial activity: Under the well diffusion method, zone of inhibition was observed for CuO-NPs at a concentration of above 400 µg mL^{-1} (Fig. 4). At 500 µg mL^{-1} concentration of CuO-NPs, zone of inhibition was measured as 14.3 mm whereas it was 15.2 mm for ketoconozole.

Treatment of *M. furfur* with CuO-NPs resulted in a decrease in yeast growth in the OD (Optical Density) medium. The MIC_{50} value of CuO-NPs was determined as <100 µg mL^{-1}. Higher concentrations of ketoconazole (100-1000 µg mL^{-1}) were effective on the yeast growth, resulting in 100% reduction of the optical density in OD medium (Fig. 5). The MIC is defined as the lowest concentration of NPs that inhibits the growth of a microorganism.

DISCUSSION

This study has been conducted to form copper oxide nanoparticles through actinomycetes and its antidandruff

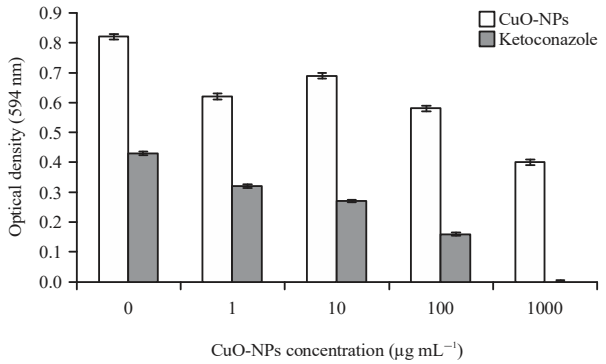

Fig. 5: Effect of CuO-NPs on the growth of *Malassezia furfur*

activity against *M. furfur*. Through *in vitro* experiments, the antidandruff efficacy of synthesized CuO-NPs against *Malasezia furfur* MTCC 1374 was investigated. The results revealed that nanoparticles size of 91 nm were obtained and CuO-NPs at a concentration of 500 μg mL^{-1} has significantly inhibited the growth of *M. furfur* at $p<0.05$ level. MIC values were determined using the positive control ketoconozole. Significant activity of ketoconazole nanoparticles against *M. furfur* (26 mm) was observed[30]. In another study, antimalassezial activity of ZnO super structures were found to have MIC values ranging[31] from 8-125 μg mL^{-1}. Antidandruff activity of silver nanoparticles from *Solanum trilobatum* was reported by Pant *et al*.[32] Similarly, in situ capped silver nanoparticles prepared using *Acacia* and *Aegle marmelos* leaf extracts had exhibited anti-*Malassezia* activity[33].

Allaker[34] showed that CuO-NPs in a concentration of 100-5000 μg mL^{-1} had no toxic effect on human cells. The micro- and nano-copper oxide up to 50 μg mL^{-1} showed no genotoxic and cytotoxic effects on Hela cells[35]. During the biosynthesis of copper nanoparticles, oxidation or reduction is the main reaction which occurs[36]. According to the study, this is the first report about the growth inhibition of *Malassezia furfur* by actinomycetes mediated CuO-NPs. These findings are important because this yeast has been associated with dandruff in humans. Summarizing, concentrations of the actinomycetes mediated CuO-NPs higher than 75 μg mL^{-1} inhibit the growth of the *Malassezia* significantly. The antidandruff activity of actinomycetes mediated CuO-NPs could be utilized as formulations in antidandruff shampoos to control dandruff in humans. At the same time, the mechanisms in which the CuO-NPs inhibit the growth of *M. furfur* need to be explored to utilize the formulation to control other species of *Malassezia*.

CONCLUSION

CuO-NPs were successfully synthesized by chemical reduction of copper ions in the presence of actinomycetes. Formation of nanoparticle sized copper oxide nanoparticles indicated the use of *Streptomyces* in green synthesis method. The mechanism of growth inhibition induced by the green-synthesized CuO-NPs was elucidated by zone of inhibition.

SIGNIFICANCE STATEMENT

This study proposes green-synthesis method for the production of CuO-NPs using actinomycetes with high anti-malassezial activity. Thus, this nanomaterial could be useful for controlling *Malassezia furfur* that affects humans globally.

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