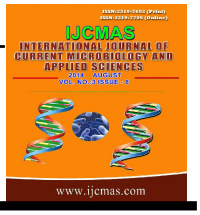
Int.J.Curr.Microbiol.App.Sci (2014) 3(9) 374-383

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 9 (2014) pp. 374-383**
http://www.ijcmas.com



Original Research Article

Biological synthesis of Copper nanoparticles using *Pseudomonas fluorescens*

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Keywords

Copper nanoparticles (CuNPs), *Pseudomonas fluorescens*, (SEM), energy dispersive spectroscopy (EDS), (TEM)

A B S T R A C T

The development of simple, cost effective and reliable methods for synthesis of nanomaterials is being emphasized globally. Biosynthesis methods, employing microorganisms, have emerged as an eco-friendly, clean and viable alternative to chemical and physical methods. The present study reports the biosynthesis of copper nanoparticles (CuNPs) using cell-free culture supernatant of non-pathogenic bacteria *Pseudomonas fluorescens*. The CuNPs were characterized using UV-Visible spectroscopy, SEM-EDS, TEM and SAED (selected area electron diffraction). It was found that the average particle size was 49 nm with spherical and hexagonal shapes. The influencing parameters such as pH, concentration of copper, volume of cell-free supernatant used and reaction time were studied. Neutral pH, 318.4 ppm Cu and 10 mL supernatant were optimal for NP production. Well-defined CuO NP formation occurred after 90 min incubation. NPs remained stable in aqueous solution with increasing time. It was established that the presence of some heat labile molecules released by bacteria in the cell-free supernatant leads to the reduction and formation of CuNPs which have to be identified in future.

Introduction

The synthesis and use of metal nanoparticles have gained consideration due to their unique electrical, optical, catalytic and magnetic properties [1-5] which are different from bulk materials. In recent times, synthesis of inorganic nanoparticles has been demonstrated by many chemical and physical methods. But the importance of biological synthesis is being realized globally as chemical methods are toxic, costly and non-ecofriendly [6]. It is, therefore, important to develop synthetic strategies which are simple, cost-effective, environment friendly and easy to scale. Hence, importance is being given to develop eco-friendly green route for the production of metal nanoparticles using biological systems.

It is well established that when microbes are kept in toxic metal environment, they evolve mechanism to survive in harsh conditions by transforming toxic metal ions into their corresponding non-toxic forms like metal sulfide/oxides [7]. The details of mechanisms involved in nanoscale transformation are not well established. A wide group of biological resources like

bacteria, yeasts, fungi, algae and plants can be used for the synthesis of nanoparticles. Among all biological systems used till now, bacteria have acquired significant attention [8] as they are easy to culture, produce extracellular nanoparticle. Also, it requires mild experimental conditions like pH, temperature, have easy downstream processing and short generation time for nanoparticle synthesis [9].

Among transition metals, interest is being shown to copper nanoparticles because of its unique properties and potential applications as catalysts [10], lubricants [11], electronic materials [12], thermal transfer nanofluids [13, 14], nanocomposite coatings [15] and in optical devices [16]. Copper nanoparticles (CuNPs) are considered as candidates for antimicrobial applications as biocides and antibiotic treatment. These have been shown to hinder the growth of bacteria like *Escherichia coli* and *Bacillus* subtilis [17]. Nanocopper can be used in the construction of biosensors and as contraceptive agent. The present work describes the synthesis of Cu nanoparticles using a non-pathogenic *Pseudomonas fluorescens* and characterized by UV-Visible spectroscopy, scanning electron microscopy (SEM-EDS), transmission electron microscopy (HR-TEM) and selected area electron diffraction (SAED) techniques.

Materials and Methods

Culturing of *Pseudomonas fluorescens*

Pseudomonas fluorescens MTCC 103 was obtained from MTCC, Chandigarh, India and grown in F-Base media (King's B media) which is specific for *P.fluorescens*. F-Base media contains (g/l): Peptone, 20; dipotassium hydrogen phosphate, 1.5; magnesium sulphate, 1.5; Agar, 15; glycerol, 10 mL pH 7.2. 1×10^{-3} dm^3 inoculum was transferred into 50×10^{-3} dm^3 F- Base media and maintained at 30ºC in an incubator shaker set at 150 rpm.

Biosynthesis of CuNPs by *P.fluorescens*

Copper nanoparticles were synthesized using *P.fluorescens* by treating copper sulphate ($CuSO_4$) solution independently with cell pellet and cell free supernatant. After 48 h, the culture was centrifuged at 7500 rpm for 20 min at 30ºC and the cell pellet as well as cell-free culture supernatant was recovered and used independently.

1 g of biomass was suspended independently in 20 mL of 318, 750 and 1000 ppm ($CuSO_4$) solution. Supernatant was collected, centrifuged at 10,000 rpm for 20 min at 30 ºC and investigated for production of CuNP. Different volumes of cell-free supernatant were added to various concentrations of $CuSO_4$ solution and placed in an incubated shaker machine for 24 to 48 h at 30 ºC, 150 rpm. They were then investigated for CuNP production by UV-Vis spectral analysis (400-800 nm). Cell-free spent media was also assessed for its ability to mediate synthesize of CuNPs. The control experiments were also carried out with un-inoculated media and $CuSO_4$ solution.

Characterization of copper nanoparticles

The preliminary detection for CuNP formation was observed by visual color change. CuNPs were further characterized by UV-Vis spectroscopy, SEM and HR-TEM techniques. Reduction of Cu^{2+} in the prepared mixtures was monitored by UV-Vis spectral analysis from 400 to 800 nm using UV-Vis spectrophotometer (Shimadzu, UV 1601). The suspension of CuNPs were dried and subjected to SEM-EDS using a JEOL MODEL JSM 6360 SEM. The morphology and size of CuNPs was studied by TEM. For this, an aliquot of

an aqueous suspension of CuNPs was transferred onto an amorphous carbon coated copper grid, dried and analyzed using a Jeol JEM 2100 TEM operated at voltage of 80 kV.

Effect of F-Base media components on CuNP formation

Cell-free supernatant, F-Base media, peptone, dipotassium hydrogen phosphate and hepta hydrated magnesium sulphate were added independently to 1000 ppm Cu and incubated for 48 h at 30 ºC and allowed to undergo a reaction in a orbital shaker, agitated at 150 rpm. The supernatant was collected and centrifuged at 10,000 rpm for 20 min at 30 ºC. The clear solution was analyzed for CuNP formation using UV-Vis spectrophotometer.

Effect of pH on CuNP production

To study the effect of pH on CuNP production, cell-free supernatant was exposed to $CuSO_4$ (1000 ppm Cu) at pH 3, 5, 7 and 9 and incubated. Supernatant was collected to check for CuNPs by UV-Vis analysis (400-800 nm).

Effect of copper concentration on NP production

10 mL of cell-free supernatant was added to 40 mL of 318, 750 and 1000 ppm Cu solution, respectively. The mixture was incubated as above and supernatant was collected and analyzed for CuNPs by UV-Vis analysis.

Effect of cell-free supernatant volume on CuNP formation

The minimal volume of cell-free supernatant necessary for CuNP formation was estimated at different time intervals. Different volumes (1-10 mL) of cell-free supernatant were added to 10 mL of 318 ppm Cu solution and incubated. Supernatants were collected at 16 h and 41 h and analyzed for CuNP production by UV-Vis analysis.

Effect of reaction time on nanoparticle production

The cell-free supernatant was added to 318 ppm Cu solution and incubated. Supernatants were collected at different time intervals. Absorbance values were recorded at 570 and 610 nm which corresponds to CuNP and CuO NPs respectively [18, 19]. To confirm that nanoparticles are formed by biological reduction due to some enzymes/protein component, a part of cell-free supernatant was heated at 100°C for 30 min and examined for CuNP as above.

Results and Discussion

UV-Vis analysis of CuNP biosynthesis

When cell-free supernatant of *P.fluorescens* was added to $CuSO_4$ solution and incubated for 48 h, the reaction mixture's color changed from blue to dark green (Fig. 1a) indicating the formation and oxidation [20] of CuNPs. Similar appearance of a dark green color solution on addition of 5 mM $CuSO_4$ to a flask containing *Morganella* sp. indicated nanoparticle production [21]. Addition of various concentrations of $CuSO_4$ solution to pellet did not reveal any color change (Fig. 1b).

The UV-Vis absorption spectrum of this solution exhibits a distinct absorption peak in the region of 550-650 nm (Fig. 2) which can be attributed to Cu nanoparticles [22]. The exact position of SPR band may shift depending on individual particle properties including size, shape and capping agents [23].

Characterization of CuNPs by SEM and TEM

SEM analysis indicated that CuNPs were formed as aggregates and had variable morphology (Fig. 3a). Exact shape could not be identified through SEM due to lesser resolution. EDS analysis showed that 11% of copper and 24% of oxygen were present in nanoparticle (Fig. 3b). The appearance of other elements was may be due to media components or other biomolecules secreted by the bacteria.

TEM was carried out to analyze the shape and size of CuNPs. Fig. 4a shows TEM images of copper nanoparticles obtained after 48 h of reaction of cell-free supernatant with $CuSO_4$. The particle size ranged between 20-80 nm with presence of spherical and hexagonal nanoparticles. Selected area electron diffraction (SAED) pattern (inset Fig. 4a) of formed nanoparticles confirmed that copper nanoparticles were formed as Cu $(OH)_2$. SAED spots confirms the presence of [002] and [112] planes. It is revealed from Fig. 4b, the average particle size of the produced CuNPs are in the range of 30-40 nm. As seen in Fig. 5, TEM-EDS confirmed that copper is present in sample compared to grid.

Effect of media components on formation of CuNPs

Shivaji and coworkers [24] reported synthesis of AgNPs from psychrophilic bacteria using cell-free supernatant as well as media components. Intensity of peak observed was more in former case indicating that bacterial culture supernatant produces more amount of NP compared to media. This indicates that NP production may not be a biosynthetic process but a chemical conversion and presence of a factor in cell-free supernatant stabilizes nanoparticles. We observed a similar trend for the formation of CuNPs (Fig. 6). When uninoculated F-Base media was added to $CuSO_4$ solution, peak at 624 nm appeared. Peptone gave peak at 636 nm. However, intensity of peak observed for peptone and F-Base media was less significant when compared to cell-free supernatant with $CuSO_4$ at 567 nm. Both peptone and media showed absorbance at 700 nm which is not characteristic of CuNPs.

Effect of pH on CuNP production

It is said that altering the pH can help in shape and size control of nanoparticles [25]. Peaks observed at acidic pH of 3 and 5 were not characteristic of CuNP. Alkaline pH gave a sharp peak at 561 nm indicating presence of larger-sized particles or aggregates. Increase or decrease of alkalinity led to aggregation or distortion of Ag particles was also reported in *E. coli* [25]. In our case, at pH 7, a characteristic peak observed at 653 nm confirms the CuNP production. Fig. 7 indicating that neutral pH is optimal for the NP formation. Gold nanoparticles are synthesized at pH 7 using *R. capsulate* reported earlier [26].

Effect of $CuSO_4$ concentration and cell free supernatant on CuNP production

To understand the influence of concentration of $CuSO_4$, different concentrations of $CuSO_4$ (318.4, 750 and 1000 ppm Cu) were used as shown in Fig. 8. Control experiments did not reveal any characteristic peak in the region of 550-650 nm ascribed as CuNPs. When cell-free supernatant was added to 750 and 1000 ppm Cu, absorption peaks were observed in the region above 700 nm. On addition of 10 mL of cell-free supernatant to 318.4 ppm Cu solution, a characteristic peak was observed, suggesting 10 mL supernatant was sufficient to reduce copper ions in

solution to form copper nanoparticles. Further increase in volume of cell-free supernatant (40 mL) to 1000 ppm copper solution, leads to peak sharpening with aggregation of CuNPs.

Effect of cell free supernatant volume on CuNP formation

In order to understand the volume of cell free supernatant required for the effective production of NPs, different volume of supernatant were used at different time intervals. When samples were taken at 16 h with a lowest volume (1 mL), peak for CuNP was not seen whereas addition of 2 to 10 mL of cell-free supernatant gave peaks between 590 to 630 nm. The characteristic peak at 610 nm in Fig. 9a, suggested the formation of CuNPs, as reported in the literature [23, 27, 28]. On further increase in time of incubation, higher volume of cell-free supernatant (10 mL) peak showed better stability as compared to other volumes. It might be due to presence of higher quantity of factors which are responsible for stabilizing CuNPs (Fig. 9b) indicating that NPs remain stable in solution for longer time.

Effect of reaction time on nanoparticle production

Since time is an important aspect that supports nanoparticle synthesis and stability. It was seen that absorbance at 610 nm increased gradually upto 90 min then it started decreasing. This denotes that CuO NP formation occurs and with an increase in time, size reduction takes place. CuNP synthesized by chemical reduction method using vitamin C as protective agent showed that reaction time of upto 60 min led to formation of well defined NPs [29]. Further confirmation that nanoparticles are produced due to biological reduction was done by monitoring the reaction of 318.4 ppm Cu solution with both untreated and heat-treated cell-free supernatants at 15 min intervals till 250 min.

Heat-treated cell-free supernatant did not give absorbance at 570 or 610 nm which means that copper/copper oxide nanoparticle production did not take place in this case. This confirms that nanoparticle formation is indeed due to reduction of copper ions by heat sensitive factors which are released by the bacteria into cell-free supernatant.

Fig. 1 a Color changes from blue to green for supernatant b No color change for pellet

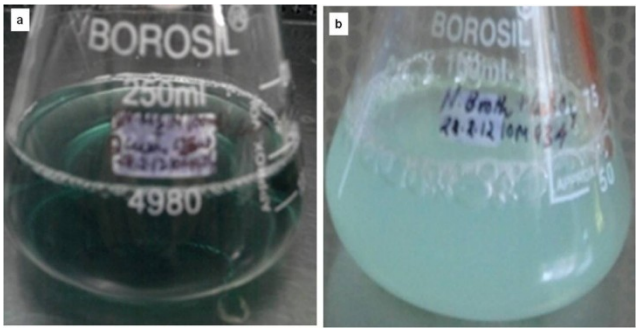


Fig. 2 Absorption spectra of copper nanoparticles synthesized by culture supernatant of *P. fluorescens*

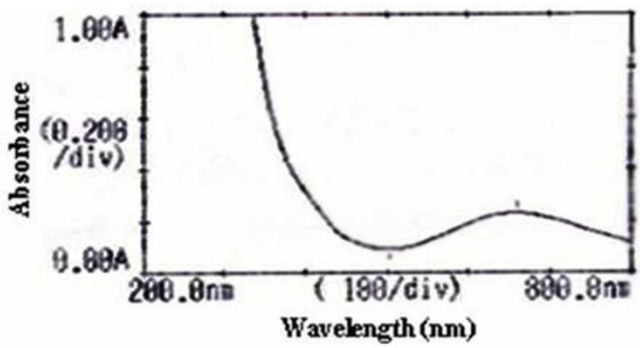


Fig. 3 a SEM image of copper nanoparticles b EDS analysis of nanoparticles

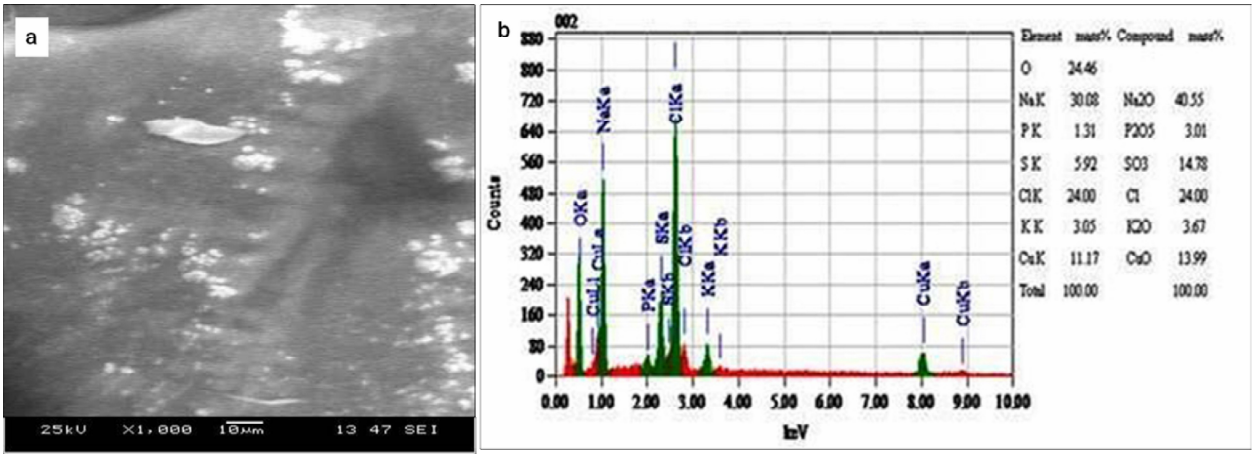


Fig. 4 a TEM micrograph and SAED pattern (inset) for CuNPs b Particle-size distribution of nanoparticles produced

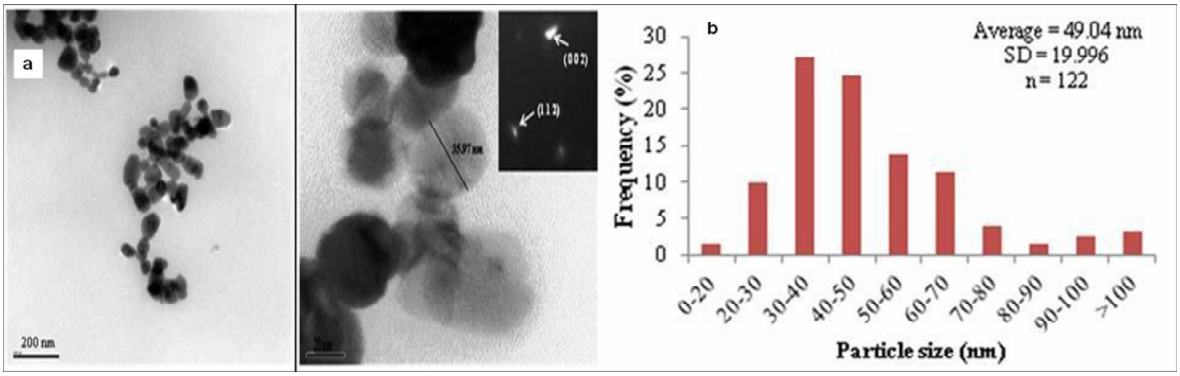


Fig. 5 TEM-EDS analysis of copper nanoparticles

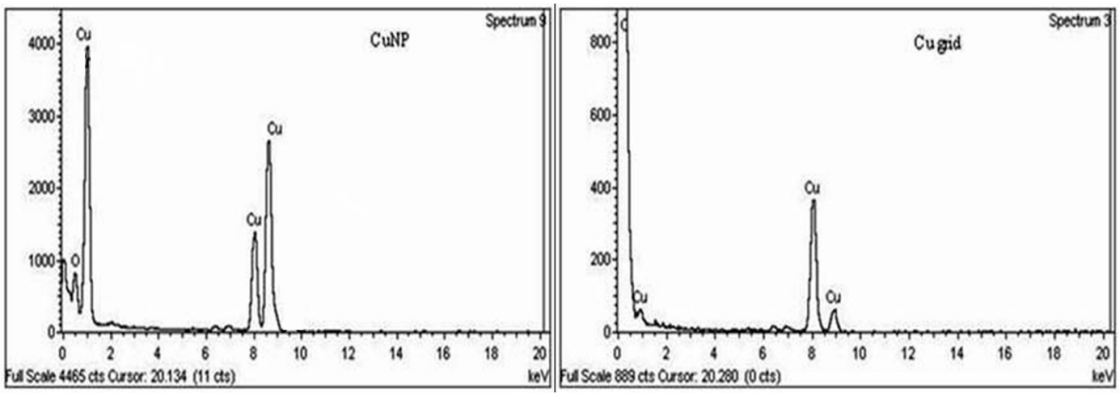


Fig. 6 CuNP synthesis by F-Base media components and cell-free supernatant (A: uninoculated F-Base media; B: cell-free supernatant; 1000 ppm Cu added to C: cell-free supernatant, D: Peptone, E: F-Base media)

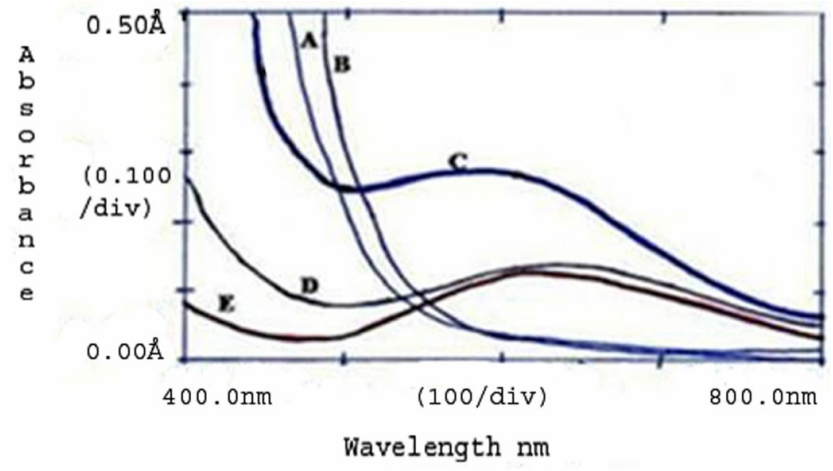


Fig. 7 Effect of different pH on CuNP formation (Cell free supernatant added with 1000 ppm Cu and pH adjusted to A: pH 3, B: pH 5, C: pH 7, D: pH 9)

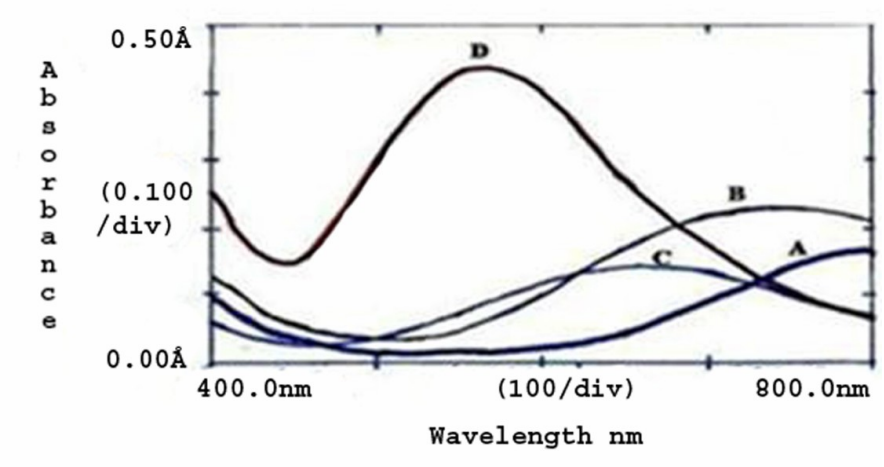


Fig. 8 Effect of copper concentration on NP production (Cell-free supernatant added to different concentrations of CuSO4 solution (A: 318.4 ppm, B: 750 ppm, C: 1000 ppm Cu controls; 10 mL cell-free supernatant added with 40 mL of D: 318.4 ppm, E: 750 ppm, F: 1000 ppm Cu; G: 40 mL cell-free supernatant with 20 mL 1000 ppm Cu)

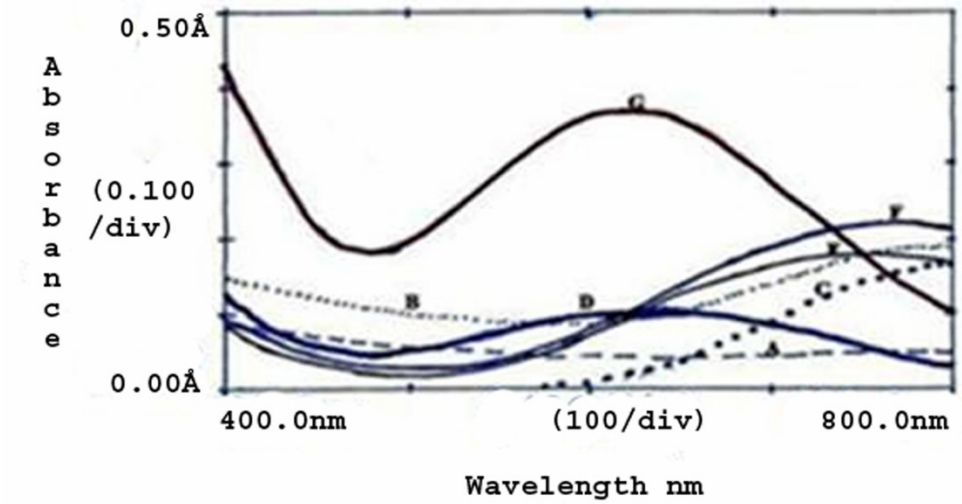
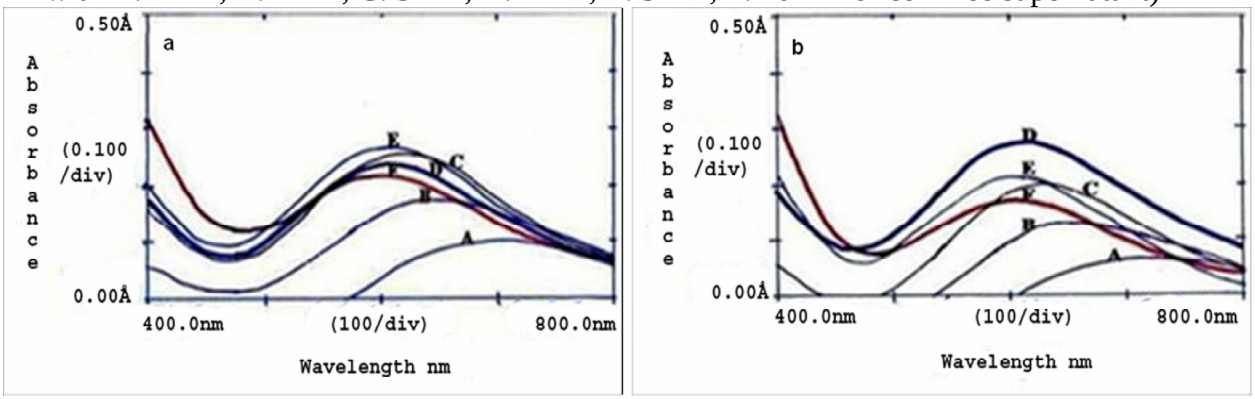


Fig. 9 a CuNP formation with varying volume of cell-free supernatant at 16 h b CuNP formation with varying volume of cell-free supernatant at 41 h (10 mL 318.4 ppm Cu added with A: 1 mL, B: 2 mL, C: 3 mL, D: 4 mL, E: 5 mL, F: 10 mL of cell-free supernatant)



Acknowledgement

The authors thank PSG College of Technology for facilitating the successful completion of this work.

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