



Internationally indexed journal

Indexed in Chemical Abstract Services (USA), Index copernicus, Ulrichs Directory of Periodicals, Google scholar, CABI ,DOAJ , PSOAR, EBSCO , Open J gate , Proquest , SCOPUS , EMBASE ,etc.



Rapid and Easy Publishing

The "International Journal of Pharma and Bio Sciences" (IJPBS) is an international journal in English published quarterly. The aim of IJPBS is to publish peer reviewed research and review articles rapidly without delay in the developing field of pharmaceutical and biological sciences



Pharmaceutical Sciences

- Pharmaceutics
- Novel drug delivery system
- Nanotechnology
- Pharmacology
- Pharmacognosy
- Analytical chemistry
- Pharmacy practice
- Pharmacogenomics



Biological Sciences

- Polymer sciences
- Biomaterial sciences
- Medicinal chemistry
- Natural chemistry
- Biotechnology
- Pharmacoinformatics
- Biopharmaceutics
- Biochemistry
- Biotechnology
- Bioinformatics
- Cell biology
- Microbiology
- Molecular biology
- Neurobiology
- Cytology
- Pathology
- Immunobiology

**Indexed in Elsevier Bibliographic Database
(Scopus and EMBASE)**

SCImago Journal Rank 0.288

Impact factor 2.958*

Chemical Abstracts
Service (www.cas.org)



A division of the American Chemical Society

CODEN IJPBJ2



Elsevier Bibliographic databases (Scopus & Embase)

SNIP value – 0.77

SJR - 0.288

IPP - 0.479

SNIP – Source normalised impact per paper

SJR – SCImago Journal rank

IPP – Impact per publication

Source – www.journalmetrics.com

(Powered by scopus (ELSEVIER))



LUND
UNIVERSITY



JACKSONVILLE STATE UNIVERSITY

Jacksonville State University
Houston Cole Library
USA (Alabama)



UNIVERSITY OF
OXFORD

Oxford, United Kingdom

INDEX COPERNICUS
INTERNATIONAL

*And indexed/catalogued in
many more university*



*Instruction to Authors visit www.ijpbs.net

For any Queries, visit "contact" of www.ijpbs.net

**BIOSYNTHESIS AND CHARACTERIZATION OF COPPER
NANOPARTICLES FROM ENTEROCOCCUS FAECALIS****ASHAJYOTHI C , KUDSI JAHANARA AND KELMANI CHANDRAKANTH R***

**Department of Biotechnology, Gulbarga University, Gulbarga-585 106
Karnataka, India.*

ABSTRACT

This investigation discuss about biogenic synthesis of copper nanoparticles from standardized *Enterococcus faecalis* culture supernatant with 100mM copper sulfate (v/v ratio) solution. The organisms show a unique potential in environmentally friendly production of nanoparticles with different sizes. The extracellular presence of copper nanoparticles was characterized by means of UV-Visible spectrum, Field Emission Scanning Electron Microscopy (FeSEM), Energy-dispersive X-ray (EDX) and Nanoparticle Tracking Analysis (NTA). FeSEM analysis studies confirmed the formation of well-dispersed copper nanoparticles with average particle size was formed to be in the range of 20 to 90nm. NTA analysis studies also confirmed the concentration of the particles in the range of 6.52×10^{10} particles/ml. We also studied the antibacterial effect of biogenic copper nanoparticles against multidrug resistant pathogens like *E. coli*, *Klebsiella pneumoniae*, Methicillin Resistant *Staphylococcus aureus* (MRSA) and with standard cultures by modification of the agar well diffusion method.

KEY WORDS: Copper nanoparticles, *Enterococcus faecalis*, Copper sulfate, Multidrug resistant pathogens.

**CHANDRAKANTH R**

Department of Biotechnology ,Gulbarga University,
Gulbarga-585106. Karnataka. India.

*Correspondingauthor

INTRODUCTION

Nanobiotechnology is an enabling technology that deals with nano-meter sized materials in different field of science such as biotechnology, nanotechnology, physics, chemistry and material science ¹. Beside many physical and chemical methods which have been developed for preparing metallic nanoparticles, nanobiotechnology also serves as an important method in the development of clean, nontoxic, and environmentally friendly procedures for the synthesis and assembly of metallic nanoparticles. Biosynthesis of metallic nanoparticles using microorganisms are a fabulous and emerging eco-friendly science of well-defined sizes, shapes and controlled monodispersity. These nanoparticles have unique catalytic, electronic and optical properties different from the metallic particles ^{2, 3}. Till date, the research in the field of biosynthesis has been mainly focused on Ag and Au nanoparticles, and there have been very few reports on the synthesis of Cu/CuO nanoparticles ⁴. The few papers in the literature on synthesis of Cu nanoparticles have been able to synthesize them in their oxide form ^{5, 6}. The green synthesis of metallic nanoparticles includes use of biological agents such as bacteria, fungi, actinomycetes, yeast and plants ⁷. In green nanotechnology, different microorganisms produce inorganic materials, either intracellularly or extracellularly with properties similar to chemically synthesized materials ⁸. Usha et al., (2010) reported a green synthesis of copper oxide by *Streptomyces* Sp for development of antimicrobial textiles which can be useful in hospitals to prevent or to minimize infection with pathogenic bacteria ⁹. For development of antimicrobial textiles which can be useful in hospitals to prevent or to minimize infection with pathogenic bacteria ¹⁰. Singh et al., (2010) reported biological synthesis of copper oxide nanoparticles using *Escherichia coli* with a variable size and shapes ¹¹. Yoon et al., (2007) reported the antibacterial effects of silver and copper nanoparticles using single representative strains of *E. coli* and *Bacillus subtilis*, where the copper nanoparticles demonstrated superior antibacterial activity compared to the silver nanoparticles ¹². Our aim of the current study is synthesis of copper nanoparticles

from *Enterococcus faecalis* and their antibacterial activity was studied against different multi drug resistant human pathogens and standard bacterial cultures.

MATERIALS AND METHODS

i. Standardization of Bacterial culture supernatant

Enterococcus faecalis (Non pathogenic) were collected from Medical Biotechnology and Phage Therapy Laboratory (MBPT), Department of Biotechnology, Gulbarga University, Gulbarga. The culture was inoculated on Bile esculin azide agar medium and incubated at 37°C, pH: 7.2 for 24hr pure culture. Single isolated colony of bacterial culture was inoculated to Luria-Bertani broth and incubated at 37°C for 6hrs. Optical density of 6hrs pure culture was taken at 600nm absorbance and serially diluted in 0.9% saline to determine Colony forming unit (CFU/ml) on Nutrient Agar media. 100µl of standard overnight culture was inoculated in to Luria-Bertani broth, incubated for 24hr and centrifuged at 10,000 rpm for 10 minute and supernatant was used for copper nanoparticle synthesis.

ii. Synthesis of Copper nanoparticles

Bacterial supernatant was added separately to the reaction vessel containing 100 mM Copper sulfate solution (v/v) and control (without Copper sulfate). The reaction was carried out in light conditions for 24 hours, at 37°C, pH: 7.2 in rotary shaker with 120 rpm.

iii. Visual observation and Spectroscopic Characterization of Copper nanoparticles

Biosynthesized copper nanoparticles were analyzed by visual observation and by other spectroscopic and analytical techniques. The 24hr reaction medium was centrifuged at 10,000 rpm for 15min, supernatant was used for UV-Visible spectrum analysis using T90+ UV-VIS spectrophotometer at 300-800 nm and for Nanoparticle Tracking Analyzer (NTA) pattern analysis. The size, shape, concentration and chemical composition of nanoparticles were analyzed using Field

Emission Scanning Electron Microscopy (FeSEM), EDX and NTA analysis.

iv. Antibacterial activity of Copper nanoparticles

E. coli (Clinical MDR pathogen), *Klebsiella pneumoniae* (Clinical MDR pathogen), clinical pathogen Methicillin resistant *Staphylococcus aureus* (MRSA), and standard bacterial cultures like *E. coli* MTCC 9537, *K. pneumoniae* MTCC 109 and *Staphylococcus aureus* MTCC 96 were used to study the effect of antibacterial activity of copper nanoparticles by modified agar well diffusion method¹³. Clinical pathogen and standard bacterial culture were inoculated in Luria-Bertani broth and incubated at 37°C for 6hr, 100µl of each of microorganism were inoculated on Muller Hinton agar (MHA) plates; Agar wells of 5 mm diameter were prepared with the help of a sterilized stainless steel cork borer. Different concentrations of copper nanoparticles (10, 20, 30, 40, 50 and 60 µl) were taken in different wells and copper sulfate was used as control in another well, were loaded with the help of micropipette under aseptic conditions and plates were incubated at 37°C for 18 and 24 hr.

RESULTS AND DISCUSSION

For standardization and copper nanoparticle synthesis *Enterococcus faecalis* culture supernatant was used. Colony forming unit (CFU/ml) was determined. The 6hr culture show 0.99 absorbance at 600nm and 1.2×10^7 CFU/ml. This standard culture of *Enterococcus faecalis* in extracellular synthesis of copper nanoparticle was confirmed by visual observation, the medium which was characterized by the changes in color from yellowish white to dark green (Fig: 1). According to Ramanathan *et al.*, (2011) report addition of 5 mM copper sulphate solution to the flask containing *Morganella sps.* led to appearance of a dark green color solution indicating the formation of nanoparticles¹⁴. This color change indicates the synthesis of copper nanoparticles by the action of extracellular medium on copper sulfate by reducing enzymes present in the bacterial supernatant. The striking dark green color of copper nanoparticles is due to excitation of surface Plasmon vibrations on the nanoparticles.

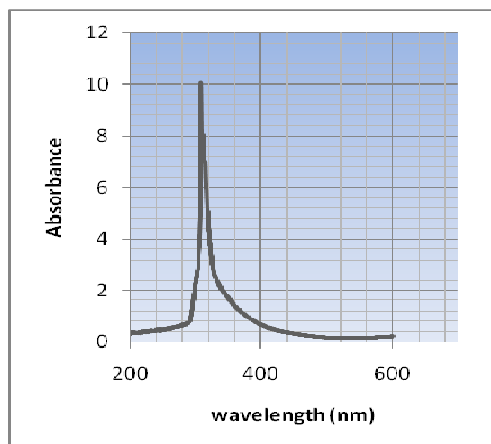
Figure 1
Visual Observation



UV-Visible Spectrum Analysis

Copper nanoparticles formed in the reaction mixture was confirmed by taking UV-Vis spectra between 300 nm to 310 nm (Fig: 2). The spectroscopic analysis showed the maximum absorbance at 306 nm indicating the presence of nanoparticles in the reaction mixture. Dark green color arises due to excitation of surface plasmon vibrations in the copper nanoparticles. According to Pavani *et al.*, (2013) copper nanoparticles synthesized from *Aspergillus species* confirms the presence of copper nanoparticles at 300nm¹⁵.

Figure 2
UV-visible spectrum (Copper nanoparticles)



Field Emission Scanning Electron Microscope (FeSEM) and EDX analysis

Field Emission Scanning Electron Microscope has been used to examine the size and shape of the nanoparticles. SEM micrographs image show copper nanoparticles, size ranging from 20-90 nm and spherical in shape (Fig: 3) Result obtained was quite similar to the result obtained in Nanoparticle Tracking Analyzer (Fig: 5 and 6). EDX (energy-dispersive X-ray) report confirms a chemical composition or contaminants for the synthesized copper

nanoparticles. The EDX spectrum gives the number of signals for contaminants in nanoparticles. Mainly it shows two types of signal peaks, one was copper atom and other peak was elemental oxygen. Results of our study prove that only oxygen atoms are the major contaminants in synthesized copper nanoparticles (Fig: 4). The signals were likely due to X-ray emission from carbohydrates/proteins/enzymes present in the cell wall of the biomass¹⁶.

Figure 3
FeSEM images of biosynthesized copper nanoparticles from *Enterococcus faecalis*

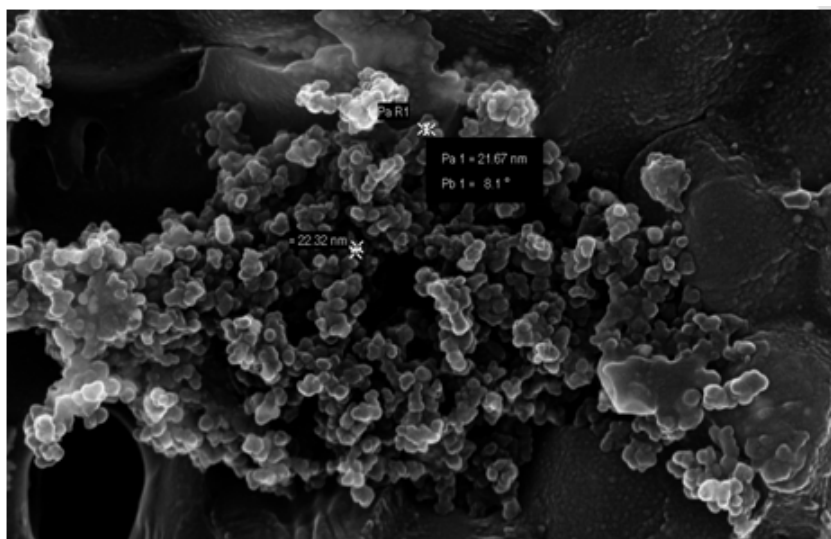
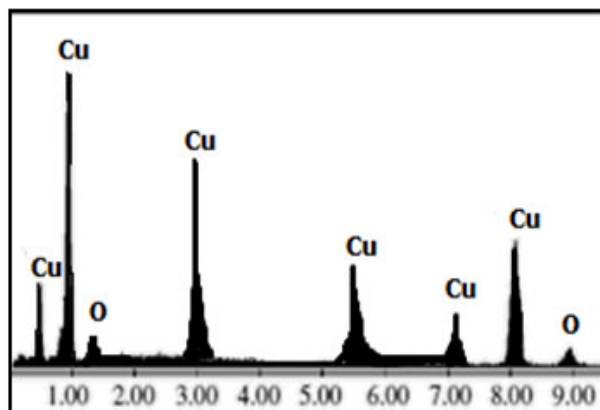


Figure4
EDX image of copper nanoparticle



Nanoparticle Tracking Analysis (NTA)

Nanoparticle Tracking Analysis is a newly developed method for visualization and analysis of nanoparticles present in liquid state. Analysis confirms the presence of copper nanoparticles sample with 6.52×10^{10} particles/ml. (Fig: 5 and 6).

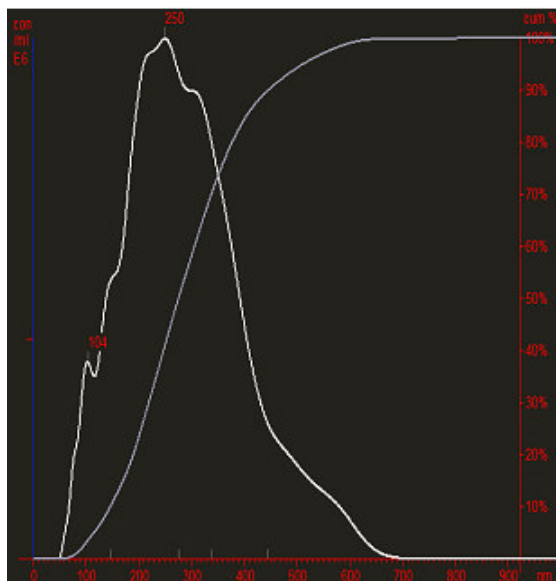


Figure 5: Particle Size / Concentration of copper nanoparticles

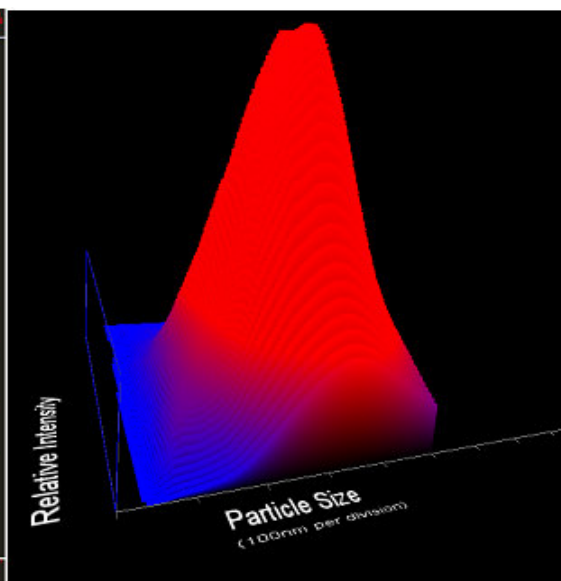


Figure 6: Particle Size / Relative Intensity 3D plot

Antibacterial activity by Agar well diffusion method

In the present work biosynthesized copper nanoparticles were examined for antibacterial activity against all the clinical pathogens and standard bacterial cultures. MDR isolates E.coli and Staphylococcus aureus showed maximum zone of inhibition of 25mm and for K. pneumonia 18mm at 60 μ l of copper nanoparticle concentrations, as compared with clinical isolated MDR, standard MTCC cultures are less sensitive against biogenic

copper nanoparticles. In comparison study of clinically isolated MDR E.coli with E. coli MTCC 9537, MDR E.coli shows more sensitive response towards copper nanoparticles for the same concentration. Clinical MDR K. pneumonia, Staphylococcus aureus and their standard cultures, K. Pneumonia MTCC 109 and Staphylococcus aureus MTCC 96 respectively, are showing equal sensitivity towards 60 μ l concentration of copper nanoparticles (Table: 1 and Fig: 7. a-f). In a comparison study between standard

antibiotics and copper nanoparticles on *E. coli* (with antibiotics Rifampicin, Piperacillin, Ceftazidime), *E. coli* MTCC 9537 (with antibiotics Ampicillin, Teicoplanin, Oxycillin), *K. pneumonia* (with antibiotics Ceftazidime, Cephalexin, Ceftriaxone) *K. pneumonia* MTCC 109 (with antibiotics Cephalexin, Ampicillin, Pencillin) and *Staphylococcus aureus* and *Staphylococcus aureus* MTCC 96 (with antibiotics Ampicillin, Methicillin, Pencillin) show weak antibacterial effect as compared to copper nanoparticles. (Table: 2). Synthesis of nanoparticles is central to research and applications in nanotechnology¹⁷. Copper oxide nanoparticles were effective in killing a range of bacterial pathogens of hospital-acquired infections. But a high

concentration of nano CuO is required to achieve a bactericidal effect¹⁸. Copper nanoparticles have a high antimicrobial activity against *B. subtilis*. This may be attributed to greater abundance of amines and carboxyl groups on the cell surface of *B. subtilis* and greater affinity of copper towards these groups. Copper ions released may also interact with DNA molecules and intercalate with nucleic acid strands. Copper ions inside bacterial cells also disrupt biochemical processes¹⁹. The exact mechanism behind the bactericidal effect of copper nanoparticles is not clear. The antimicrobial activity of nanoparticles has been studied largely with human pathogenic bacteria, mainly *E. coli* and *S. aureus*²⁰.

Table 1
Zone of inhibition of Clinical MDR strains

SI No	Clinical MDR strains	Zone of inhibition (mm)					
		10µl	20µl	30µl	40µl	50µl	60µl
1.	<i>E. coli</i>	12	16	18	21	23	25
2.	<i>E. coli</i> MTCC 9537	-	10	12	14	17	19
3.	<i>K. pneumonia</i>	09	10	11	12	14	18
4.	<i>K. pneumonia</i> MTCC 109	-	10	13	15	16	18
5.	<i>Staphylococcus aureus</i>	08	09	11	15	20	25
6.	<i>Staphylococcus aureus</i> MTCC 96	-	13	16	19	22	25

Control (100µM copper sulfate): No zone of clearance

Table 2
Comparison of zone of inhibition of Copper nanoparticle and Antibiotics against Clinical MDR strains

Study no.	Clinical Pathogenic bacterial	Zone of Inhibition in mm			
		Antibiotics (mcg)		Copper nanoparticles (µl)	
01	<i>E. coli</i>	Rifampicin	12	25 (60µl)	
		Piperacillin	10		
		Ceftazidime	10		
02	<i>E. coli</i> MTCC 9537	Ampicillin	-	19 (60µl)	
		Teicoplanin	-		
		Oxycillin	-		
03	<i>K. pneumonia</i>	Ceftazidime	-	18 (60µl)	
		Cephalexin	-		
		Ceftriaxone	-		
04	<i>K. pneumonia</i> MTCC 109	Cephalexin	-	18 (60µl)	
		Ampicillin	-		
		Pencillin	-		
05	<i>Staphylococcus aureus</i>	Ampicillin	12	25 (60µl)	
		Methicillin	10		
		Pencillin	11		
06	<i>Staphylococcus aureus</i> MTCC 96	Ampicillin	11	25 (60µl)	
		Methicillin	09		
		Pencillin	11		

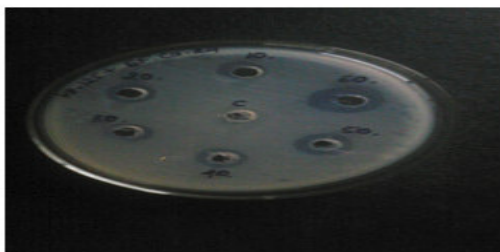
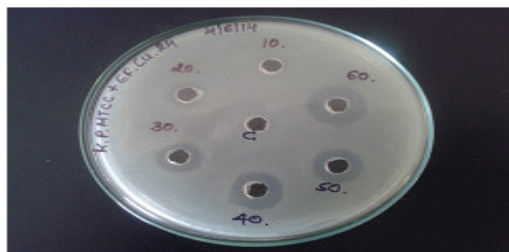
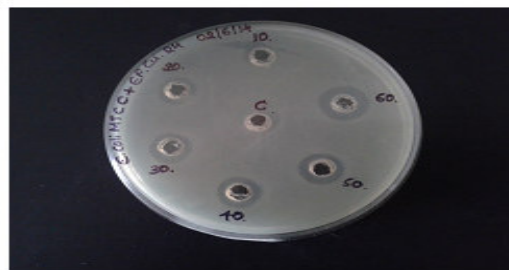
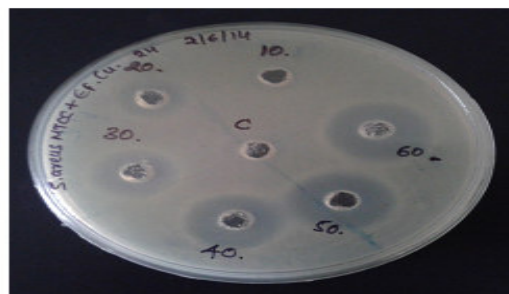
Fig 7.a. Zone of inhibition for *K. pneumonia*Fig 7.b. Zone of inhibition for *K. pneumonia* MTCC 109Fig 7.c. Zone of inhibition for *E. coli*Fig 7.d. Zone of inhibition for *E. coli* MTCC 9537

Fig 7.e. Zone of inhibition for MRSA

Fig 7.f. Zone of inhibition for *S. aureus* MTCC 96

CONCLUSION

Our study concludes that copper nanoparticles could be successfully synthesized using *E. faecalis*. The method was formed to be easy, ecofriendly and economical. UV-Visible spectroscopic analysis revealed the presence of nanoparticles in the reaction mixture. Microscopic and Nanoparticle tracking analysis confirmed the average particle size ranges from 20 to 90nm with 6.52×10^{10}

particles/ml of concentration. Copper nanoparticles have strong antibacterial activity against multidrug resistant human pathogenic bacteria's. This method proves the production of copper nanoparticles to be a potentially exciting tool for large-scale synthesis of nanoparticles and could be a strong weapon against human pathogenic bacteria.

REFERENCE

1. Soheyla Honary, Hamed Barabadi, Eshrat Gharaeifathabad, Farzaneh Naghibi, Green synthesis of copper oxide nanoparticles using *Penicillium aurantiogriseum*, *Penicillium citrinum* and *Penicillium waksmanii*. Digest Journal of Nanomaterials and Biostructures, 7(3), 999 – 1005, (2012).
2. Mubarak Ali D, Sasikala M, Gunasekaran M, Thajuddin N, Biosynthesis and characterization of silver nanoparticles using marine Cyanobacterium, *Oscillatoria willei* ntdm01, Digest Journal of Nanomaterials and Biostructures, 6(2), 385-390, (2011).

3. Rai M, Yadav A, Gade A, Current [corrected] trends in phytosynthesis of metal nanoparticles, Crit. Rev. Biotechnol. 28(4), 277-284, (2008).
4. Thakkar K N, Mhatre S S, Biological synthesis of metallic nanoparticles, Nanomed. Nanotechnol. Biol. Med. 6(2), 257-262, (2010).
5. Hasan S, Singh S, Bacterial Synthesis of Copper/Copper Oxide Nanoparticles, J. Nanosci. Nanotechnol, 8(6), 3191-3196, (2008).
6. Singh V, Patil R, Biological Synthesis of Copper Oxide Nano Particles Using Escherichia coli, Curr. Nanosci, 6(4), 365-369, (2010).
7. Thakkar K N, Mhatre S S, Parikh R Y, Biological synthesis of metallic nanoparticles, Nanomedicine, 6(2), 257-262, (2010).
8. Bäuerlein E, Angew, Bomineralization of Unicellular Organisms: An Unusual Membrane Biochemistry for the Production of Inorganic Nano- and Microstructures Chem. Int. Ed. 42, 614-641, (2003).
9. Usha R, Prabu E, Palaniswamy M, Veni C K, Rajendran K R, Synthesis of metal oxide nanoparticles by Sterptomyces species for development of antimicrobial textiles. Global J. Biotechno. Biochem, 5(3), 153-160 (2010).
10. Singh Ajay V, Patil R, Anand A, Milani P, Gade W N, Biological Synthesis of Copper Oxide Nano Particles Using Escherichia coli, Curr Nanosci 6(4), 365-369 (2010).
11. Mandal D, Bolander M E, Mukhopadhyay D, Sarkar G, Mukherjee P, The use of microorganisms for the formation of metal nanoparticles and their application, Microbiol. Biotechnol. 69(5), 485-492, (2006).
12. Yoon K, Byeon J H, Park J, Hwang J. Susceptibility constants of Escherichia coli and Bacillus subtilis to silver and copper nanoparticles, Sci Total Environ, 373, 572-575, (2007).
13. National Committee for clinical Laboratory Standards: Performance standards for Antimicrobial disk susceptibility testing. Twelfth informational supplement (M100-S12), 2000. Wayne PA: ACCLS, 2000.
14. Ramanathan R, Bhargava SK, Bansal V, Biological synthesis of copper/copper oxide nanoparticles. Chemca Conference 466. papers/466.pdf, (2011).
15. Kantabathini Venkata Pavani, Nandigam Srujana, Guntur Preethi, Tandale Swati, Synthesis of copper nanoparticles by Aaspergillus species, Letters in Applied NanoBioScience, 2(2), 110-113, (2013).
16. Mishra A. N, Bhadauria S, Gaur M. S, Pasricha R, Kushwah B.S, Synthesis of gold nanoparticles leaves of zero-calorie sweetener herb (Stevia rebaudiana) and their nanoscopic characterization by spectroscopy and microscopy. Int. J. Green Nanotechnol Phys. Chem, 1, 118-124, (2010).
17. Arabinda Mahanty, Ranadhir Bosu, Pragnya Panda, Surya Prakash Netam, Biplab Sarkar, Microwave assisted rapid combinatorial synthesis of silver nanoparticles using E.coli culture supernatant. Int J Pharm Bio Sci, 4(2), 1030 – 1035, (2013).
18. Ren G, Hu D, Cheng E. W. C, Vargas-Reus M. A, Reip P, Allaker R. P, Characterisation of copper oxide nanoparticles for antimicrobial applications. International Journal of Antimicrobial Agents, 33, 587-590, (2009).
19. Rupareli J. P, Chatterjee A. K, Duttagupta S. P, Mukherji S, Strain specificity in antimicrobial activity of silver and copper nanoparticles. Acta Biomaterialia, 4, 707-771, (2008).
20. Gajjar P, Pettee B, Britt D.W, Huang W, Johnson W.P, and Anderson A.J., Antimicrobial Activities of commercial nanoparticles against an environmental soil microbe, Pseudomonas Putida kt2440, J biol eng, 3(9), 1-13, (2009).