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BIOSYNTHESIS AND CHARACTERIZATION OF COPPER NANOPARTICLES FROM ENTEROCOCCUS FAECALIS

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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.

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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 37^0C, pH: 7.2 for 24hr pure culture. Single isolated colony of bacterial culture was inoculated *to* Luria-Bertani broth and incubated at 37^oC 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 37^0 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. pneumonia MTCC 109 and Staphylococcus aureus MTCC 96 were used to study the effect of antibacterial activity of copper nanoparticles by modified agar well diffusion method [13]. Clinical pathogen and standard bacterial culture were inoculated in Luria-Bertani broth and incubated at 37^0C 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 37ºC for 18 and 24 hr.

RESULTS AND DISCUSSION

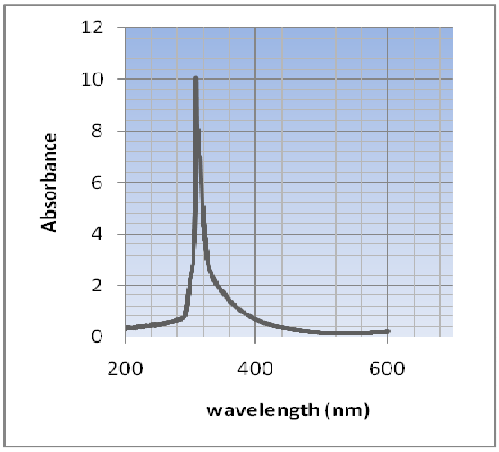
For standardization and copper nanoparticle synthesis Enterococcus faecalis culture supernatant was used. Colony forming unit (CFU/ml) was determined. The 6hr culture show 0.99 absorbance at 600nm and 1.2×10^7 CFU/ml. This standard culture of Enterococcus faecalis in extracellular synthesis of copper 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 nanoparticles [14]. This color change indicates the synthesis of copper nanoparticles by the action of extracellular medium on copper sulfate by reducting 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 300nm [15].

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 biomass [16].

Figure 3
FeSEM images of biosynthesized copper nanoparticles from Enterococcus faecalis

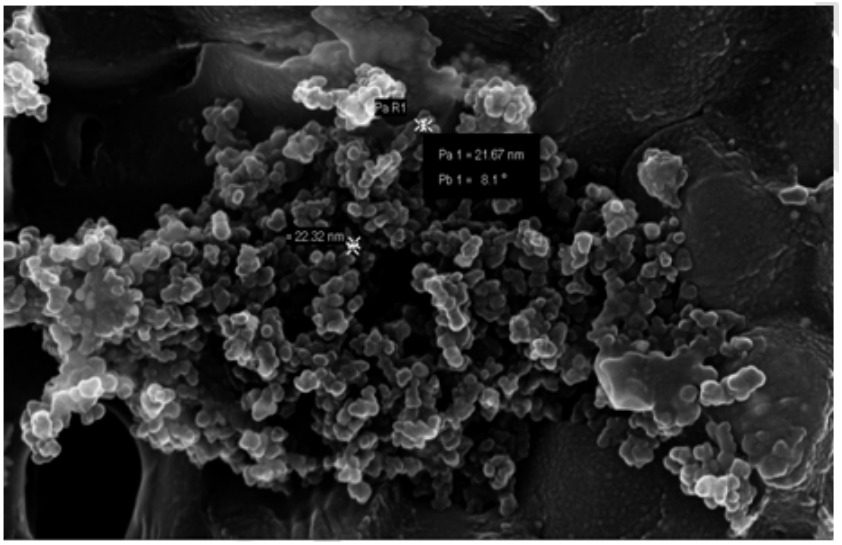
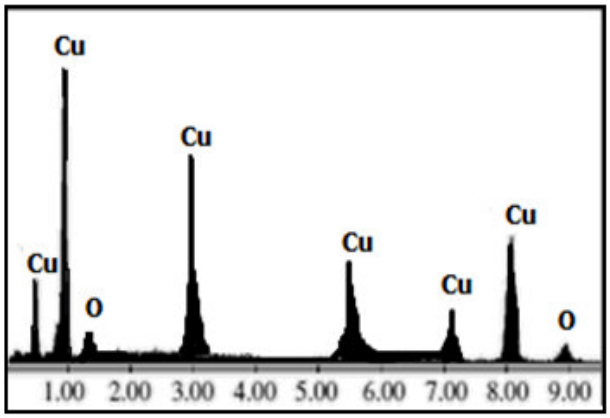


Figure4
EDX image of copper nanoparticle



Nanoparticle Tracking Analysis (NTA)

Nanoparticle Tracking Analysis is a newly developed method for visiualization 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).

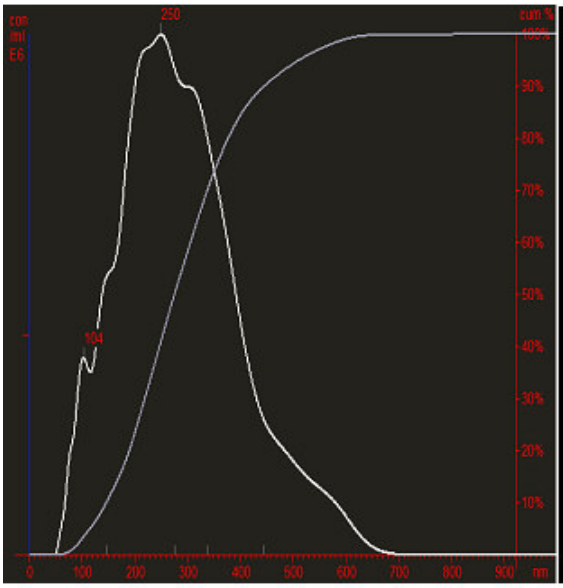


Figure 5: Particle Size / Concentration of copper nanoparticles

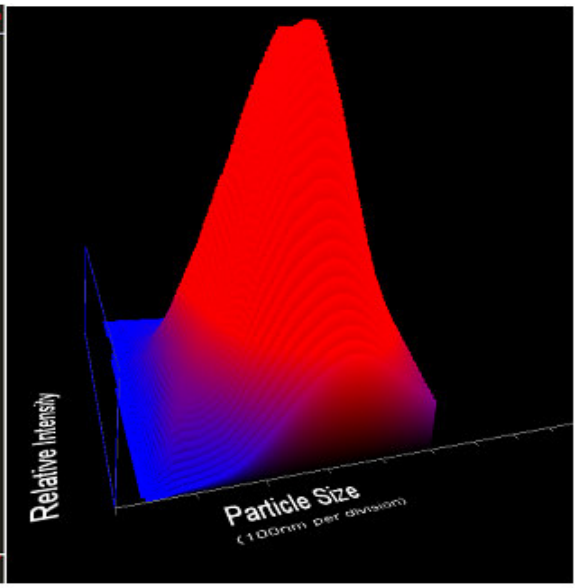


Figure 6: Particle Size / Relative Intensity 3D plot

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 60µl 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 60µl 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, Pipracillin, 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 nanaoparticles. (Table: 2). Synthesis of nanoparticles is central to research and applications in nanotechnology [17]. 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 effect [18]. 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 processes [19]. 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

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

Control (100mM 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	*E. coli*	Rifampicin	12	25 (60µl)
		Pipracillin	10	
		Ceftazidime	10	
02	*E. coli* MTCC 9537	Ampicillin	-	19 (60µl)
		Teicoplanin	-	
		Oxycillin		
03	*K. pneumonia*	Ceftazidime	-	18 (60µl)
		Cephalexin	-	
		Ceftriaxone	-	
04	*K. pneumonia* MTCC 109	Cephalexin	-	18 (60µl)
		Ampicillin	-	
		Pencillin	-	
05	*Staphylococcus aureus*	Ampicillin	12	25 (60µl)
		Methicillin	10	
		Pencillin	11	
06	*Staphylococcus aureus* MTCC 96	Ampicillin	11	25 (60µl)
		Methicillin	09	
		Pencillin	11	

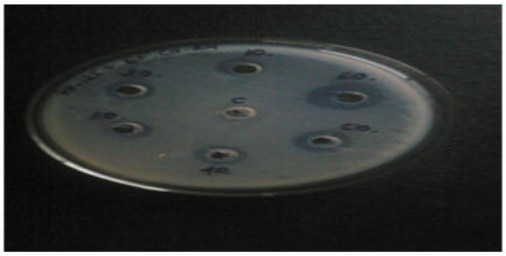


Fig 7.a. Zone of inhibition for *K. pneumonia*

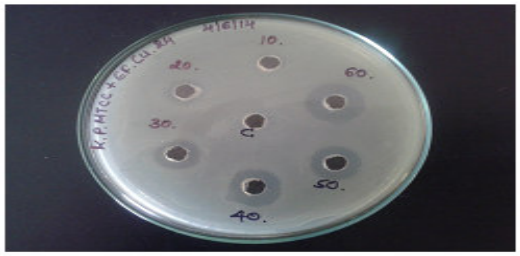


Fig 7.b. Zone of inhibition for *K. pneumonia* MTCC 109

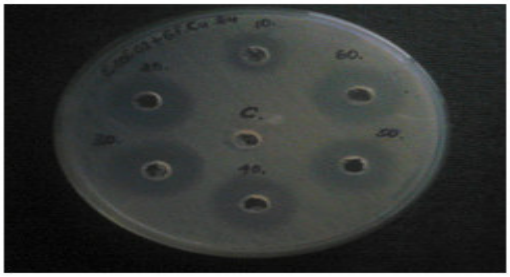


Fig 7.c. Zone of inhibition for *E. coli*

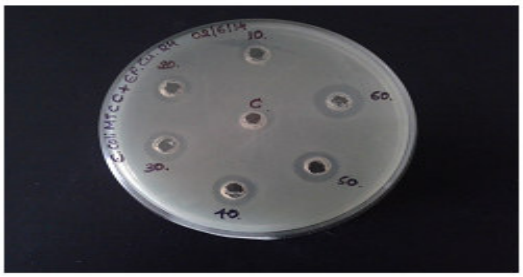


Fig 7.d. Zone of inhibition for *E. coli* MTCC 9537

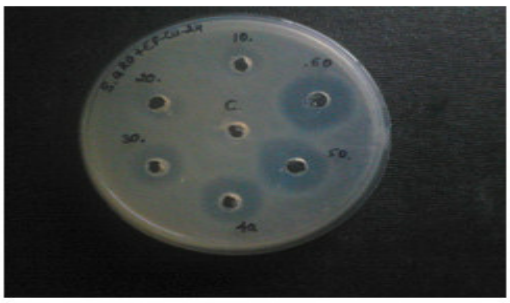


Fig 7.e. Zone of inhibition for MRSA

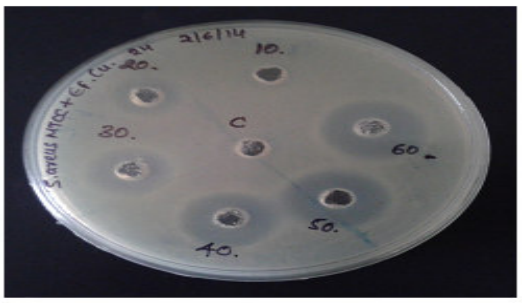


Fig 7.f. Zone of inhibition for S. aureus MTCC 96

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.

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