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Production and scrutiny of silver nanoparticles from *Corynebacterium glutamicum* ATCC13032 and antimicrobial sensitivity assessment

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Abstract

In the present study, silver nanoparticles (AgNPs) were synthesized from *C. glutamicum* and their antibacterial activity has been investigated. Dried biomass of *C. glutamicum* was challenged with aqueous Diamine silver ($[Ag(NH_3)_2]^+$) containing 1mM $AgNo_3$ at different pH conditions. Transmission electron microscopy (TEM) and Scanning electron microscope (SEM) analysis of the AgNPs were carried out. X- ray diffraction (XRD) results have shown that these nanoparticles exhibit a face-centered cubic crystal structure. Energy-dispersive X-ray (EDX) spectroscopy analysis for the confirmation of elemental silver was carried out for the detection of elemental silver. Fourier transform infrared (FTIR) spectra analysis shows that some functional groups, such as the carboxyl and amide of the proteins, were involved in the processes of bioreduction. Antimicrobial activity of the synthesized AgNPs against multi drug resistant bacteria and fungi was assessed by well diffusion method. AgNPs from *C. glutamicum* exhibited a potential antibacterial activity against the bacterial strains. The antimicrobial activity of silver nanoparticles was extremely good towards bacterial strains than the fungal strains. Thus, this study proved that the *C. glutamicum* ATCC13032 can produce AgNPs and the nanoparticles can be used as an effective antimicrobial substance.

Key words : Diamine silver, *Corynebacterium*, Silver nanoparticles, Antimicrobial activity.

INTRODUCTION

Nanotechnology is an emerging industry which is bringing us exiting new products and promises to change the way we live and work in future. Nanotechnology has dynamically developed as an important field of modern research with potential effects in electronics and medicine [1-2]. Nanobiotechnology is a new branch of science dedicated to the improvement and utilization of devices and structures ranging from 1 to 100 nm in size, in which new chemical, physical and biological properties, not seen in bulk materials, can be observed [3]. Recent survey reveals that amidest1300 NP-containing marketed products, at least 300 products (~25%) contains Ag NPs [4]. Their uniqueness arises specifically from higher surface to volume ratio and increased percentage of atoms at the grain boundaries [5].

The biosynthesis of nanoparticles as an emerging highlight of the intersection of nanotechnology and biotechnology has received increasing attention due to a growing need to develop environmentally benign technologies in material synthesis [6]. A number of approaches are available for the synthesis of silver nanoparticles for example, chemical and photochemical reactions in reverse micelles [7], thermal decomposition of silver compounds [8], microwave assisted process [9] and recently via green chemistry route [10-11] but the development of reliable technology to produce nanoparticles is an important aspect of nanotechnology. Biological synthesis process provides a wide range of environmentally acceptable methodology. Up to now, several microorganisms from bacteria to fungi have been reported to synthesize inorganic materials either intra- or extracellularly, and thus to be potentially utilized as eco-friendly nanofactories [12-13]. Biosynthetic methods have been investigated as an alternative to chemical and physical ones. These methods can be divided into two categories depending on the place where the nanoparticles or nanostructures are created as many microorganisms can provide inorganic materials either intra- or extracellularly [14].

Cell wall reduction is one of the method used to synthesize nanoparticles rapidly and in large quantities. New approaches and standardized test procedures to study the impact of nanoparticles on living cells are needed for the evaluation of potential hazards relating human exposure to nanoparticles. Cell walls of Gram positive bacteria such as *Bacillus subtilis* were found to bind with large quantities of metals than the Gram negative bacteria such as *Escherichia coli* [15]. However; some studies [16] proved that dried cells of some microorganisms could also reduce silver ions, where the processes of reduction were probably non enzymatic. The dried cells of *Bacillus megaterium* D01, *Lactobacillus* sp. A09, were capable of reducing silver ions through the interaction between silver ions and functional groups present on microbial cell wall. The ionized carboxyl group of amino acid residues and the amide of peptide chains were the main groups trapping $[Ag(NH_3)_2]^+$ onto the cell wall and some reducing groups, such as aldehyde and ketone, were involved in subsequent bioreduction for formation of nanoparticle [17].

Emergence of new resistant bacterial strains to current antibiotics has become a serious public health issue, which raised the need to develop new bactericidal materials [18]. Metal nanoparticles are having number of uses in pharmaceutical field mostly in cancer therapy, used as antimicrobial agents and also used in preparation of biosensors [19]. In particular, because of the recent advances in research on metal nanoparticles, Ag-NPs have received special attention as a possible antimicrobial agent [20-22]. Another area where silver nanoparticles have proven to be effective is in controlling and suppressing bacterial growth. It is

generally recognized that silver nanoparticles may attach to the cell wall, thus disturbing cell-wall permeability and cellular respiration. The nanoparticles may also penetrate inside the cell causing damage by interacting with phosphorus- and sulfur containing compounds such as DNA and protein. Another possible contribution to the bactericidal properties of silver nanoparticles is the release of silver ions from particles [11]. Ag-NPs exhibit potent antifungal effects on fungi, probably through destruction of membrane integrity [23] these nanoparticles can help the diabetic patients in early wound healing with minimal scars [24].

In the present paper, we report rapid production and the mechanism involved in synthesis of silver nanoparticles from *C. glutamicum* ATCC13032 and also our results support the hypothesis that Ag nanoparticles can be applied effectively in the control of microorganisms and the prevention of deleterious infections.

MATERIALS AND METHODS

Chemicals

Peptone, Beef extract, Yeast extract, Bacto tryptone, Aagar-agar, Potato dextrose, Silver nitrate *($AgNO_3$),* $NH_3 \cdot H_2O$ (25% w/w, AR), *NaOH, NaCl, HNO_3 etc.,*

Bacterial culture for silver nanoparticles production

The bacterial strain *Corynebacterium glutamicum* ATCC13032 was obtained from American Type Culture Collection (ATCC. USA). The strain was maintained at 4°C on nutrient agar slants as well as sub cultured from time to time to regulate its viability. *C. glutamicum* is a small, non-moving Gram-positive soil bacterium. It is not pathogenic, does not form spores, grows quickly, has relatively few growth requirements, has no extracellular protease secretion, and used to produce many amino acids.

Microbial Cultures to test antimicrobial sensitivity

Bacterial strains both Gram positive (*Staphylococcus aureus MTCC3160*) and Gram negative strains (*E. coli MTCC40, Salmonella enterica ser.t MTCC3917, Pseudomonas aeruginosa MTCC424, Klebsiella pneumoniae MTCC3384*) which were multidrug resistant strains, were procured from Institute of Microbial Technology (MTCC), Chandigarh, India and fungal cultures (*Candida albicans, Aspergillus niger, Aspergillus flavus and Paeciliomyces variuti)* obtained from Anantapuram Medical College Anantapuram, A.P. India, cultures were maintained at 4˚C on nutrient agar and potato dextrose agar (PDA) slants respectively.

Preparation of Diamine silver

Diamine silver complex ($[Ag(NH_3)_2]^+$) was prepared by adding dilute ammonia solution ($NH_3 \cdot H_2O$,25% w/w,AR) into aqueous solution of silver oxide (Ag_2O) until the precipitate of Ag_2O was transformed into soluble $[Ag(NH_3)_2]^+$. (Ag when treated with alkali *$AgNO_3$ forms silver oxide which in case of NH_4OH dissolves to form complex ion)*[25].

Production of biomass

C. glutamicum ATTCC13032 stock cultures were maintained by sub culturing at monthly intervals and growth conditions were optimized. Luria Broth (LB) (1% Bactotryptone, 0.5% yeast extract, 1% NaCl, pH 7.0 ± 0.2) was used for growing the organism. 250 mL of Luria broth (LB) was prepared, autoclaved at 121±1 ºC for 15minutes and inoculated with fresh batch of the bacteria, *C. glutamicum.* The culture flasks were incubated for 72hours at 30ºC with shaking at 150 rpm. After 72 hours of growth, the biomass was harvested by centrifugation at 5000 rpm for 10 minutes and the collected cell pellet was washed three times with deionized water to remove the culture medium and dried for overnight in oven at 60 ºC. Dried cells were analyzed by FTIR before bioreduction and the values were recorded. Milli-Q water is used throughout the experiment.

Biosynthesis of Ag nanoparticles using biomass of C. glutamicum ATTCC13032

Freshly prepared 15ml of diamine silver was taken into each conical flask labeled pH 2-10 and the pH values were adjusted from 2 to 10 using nitric acid and ammonium hydroxide. *C. glutamicum* dried biomass was resuspended with deionized water and added to aqueous solutions of diamine silver complex at different pH (2-10) values and made final volume to 25ml with deionized water incubated at 30°C in orbital shaker at 150rpm. Control (without the diamine silver, only biomass) was also run along with the experimental conditions. After incubation little sample was taken and centrifuged then the supernatant was assayed for the determination of silver nanoparticles formation [17].

UV-Vis *spectrophotometer* Analysis

The supernatant was diluted with deionized water (1:1) *and the absorption was measured using a UV-Vis spectrophotometer (*Thermo scientific-EVOLUTION201) equipped with matched quartz cells *at a resolution of 1nm from 200-800nm.* The spectroscopic studies were carried out to the reaction solutions at room temperature. It is generally recognized that UV-Vis spectroscopy could be used to examine the size and shape controlled nanoparticles in aqueous suspensions [26].

FTIR analysis

The biomass of *C. glutamicum* before and after the reaction was completely dried at 60°C, and the dried biomass was analyzed by Fourier transform infrared (FTIR) spectrophotometer (Thermo Nicolet Nexus 670 spectrophotometer; Washington, USA), the FTIR spectrum of the dried sample was recorded in the range 493.74 to 4003.9 cm^1 at a resolution of 4 cm^1.

Transmission Electron Microscopy (TEM)

TEM technique was employed to visualize the size and shape of Ag nanoparticles. AgNPs samples for TEM grids were prepared by sonicating the solution for 5minutes and placing few drops on the 300mesh carbon-coated copper grid and dried for the complete evaporation of water under lamp and operated at an accelerating voltage of 200 kV using EM2000Fx-II, Transmission Electron Microscope, which is a 200KV HRTEM from JEOL, Japan, to characterize the sample after usual alignment procedures. *In-situ* LCD Camera is used to record the pictures.

X- ray diffraction (XRD) measurement

The X-ray diffraction (XRD) was used to evaluate the crystal structure of films that is widely used to confirm the formation of silver. The X-ray Diffraction patterns of silver nanoparticle were recorded according to the description of Wang 2000 [27]. Sample solution drop-coated onto glass substrates were air dried and used for XRD analysis. X-ray diffraction patterns were recorded in the scanning mode on an X`pert PRO PAN analytical

instrumentoperated at 40 KV and a current of 30 mA with Cu Kα radiation. The diffraction intensities were recorded from 5.0170° to 81.9730, in 2θ angles.

Scanning electron microscope (SEM)

Scanning Electron Microscopic (SEM) analysis was done by preparing thin films of the sample on a carbon coated copper grid by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and then the film on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min. The instrument used is a JEOL 840 with Resolution at 20kV: 10nm

Energy dispersive X-ray (EDX) analysis

Energy Dispersive Spectrometry (EDS) micro-analysis is performed by measuring the energy and intensity distribution of X-ray signals generated by a focused electron beam on a specimen operating at 120kV using ESEM Quanta 200, FEI. EDX instrument. The data is used to obtain the elemental composition of the material.

Antimicrobial assay

Pure cultures of tested bacteria and fungi were sub cultured in nutrient broth and potato dextrose agar (PDA) respectively for 24hours at room temperature.

Bacteria: In this method, sterile Nutrient-Agar plate was prepared. Bacterial pathogens used in the present experiment were spread over the agar plate using sterile cotton swab. The plates were allowed to dry and a sterile well - cutter of diameter 6.0mm was used to bore wells in the agar plates. Subsequently, a 25µl of (0.025mg) nanoparticles suspension was introduced into wells of the inoculated Nutrient Agar plates and then incubated at 25^{0}C for 24 hours and measured the diameter of inhibitory zones in mm [28].

Fungi: Antifungal activity of obtained silver nanoparticles was evaluated by well diffusion method against *Candida albicans, Aspergillus niger, Aspergillus flavus and Paeciliomyces variuti* on Potato dextrose agar (PDA). PDA plates were prepared and the fresh cultures of fungi were spread over the agar plate using sterile cotton swab. The plates were allowed to dry and a sterile well - cutter of diameter 6.0mm was used to bore wells in the plates. Subsequently, a 25µl of (0.025mg) nanoparticles suspension was introduced into wells of the inoculated PDA plates and then incubated at 25^{0}C for 24 hours. After incubation, the zones of inhibition were measured. The assays were performed in triplicate and the mean values were recorded [29].

RESULTS

Production of silver nanoparticles

Production of silver nanoparticles from *C. glutamicum* ATTCC13032 with aqueous Diamine silver solution was done. The primary conformation of synthesis of nanoparticles in the medium was characterized by the changes in color from colorless to brown or deep yellow shown in Figure 1b where control (without diamine silver) showed no color formation in the culture when incubated for the same period and condition (Figure 1a).The color intensity increased with period of incubation due to the reduction in silver nanoparticles.

UV-Vis spectrophotometer analysis

Figure 2 shows the UV-Vis absorption spectra of the 96 hours old AgNPs sample at 440 nm. The spectra recorded from the *C. glutamicum* reaction vessel at different reaction times and observed increased intensity in absorption spectra of silver solution with time, indicating the formation of increased number of silver nanoparticles in the solution. The prepared aqueous solution of AgNPs showed strong absorption band between 410-440 nm which is a typical absorption band of spherical Ag nanoparticles due to their surface Plasmon.

Effect of pH on nanoparticle formation

AgNP production ability of *C. glutamicum* under various pH conditions was studied. pH was maintained from 2 to 10 in different conical flasks, conical flasks containing *C. glutamicum* and diamine silver with pH 2 and 5 was shown the color change from colorless to yellow after 15hours of incubation, color change was observed 18, 20 and 26 hours of incubation with pH 3, 4 and 6 respectively, where the pH ranges from 7-10 had taken above 60hours of incubation. Formation of nanoparticles was high at low pH range in particular at pH 2 and 5 shown in the Figure 3.

Fig 1. *C. glutamicum* Biomass containing Diamine silver before (a) and after (b) bioreduction

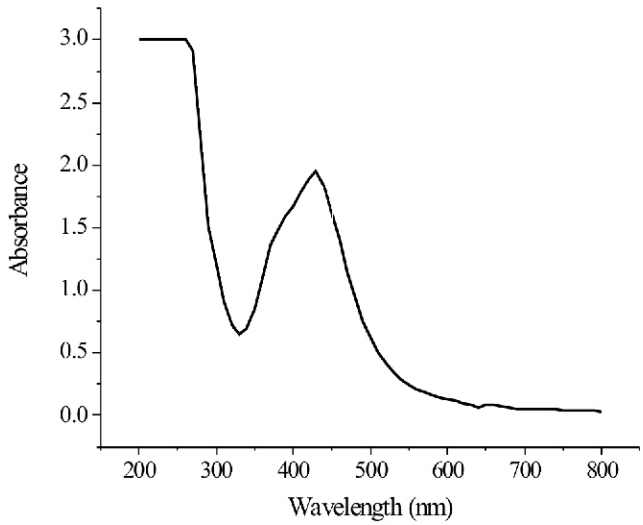

Fig 2. UV-Vis spectra of silver nanoparticles showing maximum absorbance at 440nm

Table 1. X-Ray Diffraction Peak List of Silver Nanoparticles

Pos. [°2Th.]	Height [cts]	FWHM [°2Th.]	d-spacing [Å]	Rel. Int. [%]
33.4040	111.71	0.4697	2.68251	8.96
34.0728	384.56	0.1299	2.63137	30.86
52.2141	33.48	0.2273	1.75193	2.69
64.5005	45.21	0.4180	1.44353	7.19
71.7811	52.29	0.2376	1.31397	4.20
77.0516	55.22	0.9851	1.23670	8.79

FTIR analysis

The main goal of FTIR in this study is to determine the chemical functional groups in the sample. FTIR is a powerful tool for identifying types of chemical bonds in a molecule by producing an infrared absorption spectrum that is like a molecular "fingerprint" [30]. The FTIR measurement can also be utilized to study the presence of a protein molecule in the solution, as the FTIR spectra in the 14001700 *cm1* region provides information about the presence of "*C=O*" and "*N-H*" groups [31]. The amide linkages between amino acid residues in polypeptides and proteins give rise to well known signatures in the infrared region of the electromagnetic spectrum. The positions of the amide I and II bands in the FTIR spectra of proteins are a sensitive indicator of conformational changes in the protein-secondary structure [32].

Graph-1 explains FTIR spectra for biomass of *C. glutamicum* ATTCC13032 before bioreduction, The spectrum shows the presence of three bands (Figure 4).The bands at 1652 *cm-1*(1) and 1563-(2) *cm-1* and 1398-(3) *cm-1* are due to C-N, *N-H* and COO^- stretch vibrations present in the amide linkages of the peptides, respectively and *Graph-2* explains FTIR spectra recorded after 48hrs of bioreduction, the band 1652 *cm-1* shifted to 1650 *cm-1*, the band at1398 *cm-1* moved to1386 *cm-1* with increased intensity and the intensity of the band at 1563 *cm-1* get decreased.

TEM Analysis

The well known technique for imaging solid materials at atomic resolution is Transmission Electron Microscopy (TEM). The technique was employed to visualize the size and shape of Ag nanoparticles. From the results it is observed that most of the Ag nanoparticles were spherical in shape. The TEM image (Figure 5a) shows individual silver nanoparticles as well as a number of aggregates with the size ranging 15-20nm and also electron diffraction at high resolution showing characteristic planes of silver nanoparticles in Figure 5b.

SEM analysis

The scanning electron microscopy has been employed to characterize the size, shape and morphologies of formed silver nanoparticles. The SEM image of the sample was shown in Figure 6. From The results morphology of AgNPs is more clearly seen, the particles shapes are circular and poly-dispersed and the size ranged between 15-20nm.

X-ray diffraction measurements

The crystalline nature of Ag nanoparticles was confirmed from the X-ray diffraction analysis. Figure 7 shows the XRD pattern with the diffraction peaks at 34.0728, 52.2141, 64.5005 and 77.0516 corresponding to the (111), (200), (220) and (311) planes [Table 1] which were in agreement with the face centered

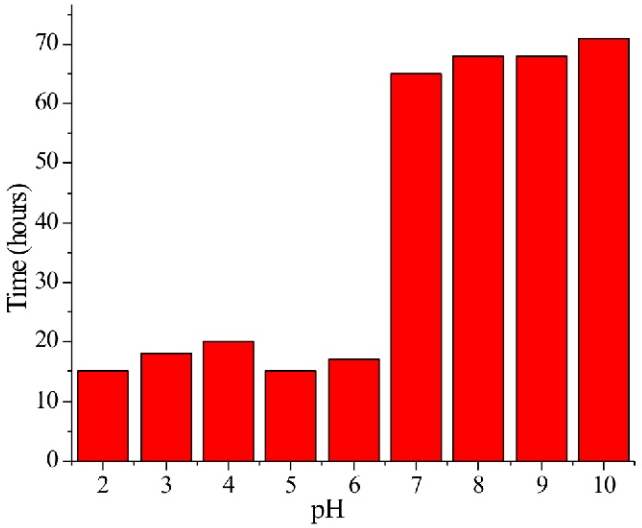

Fig 3. Showing time taken by *C. glutamicum* at different pH conditions for AgNPs production

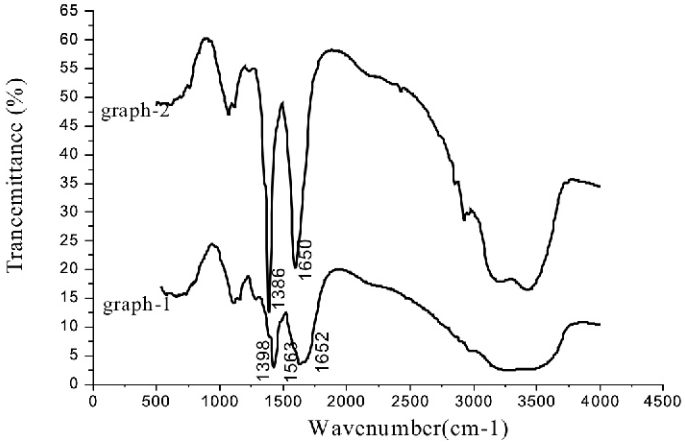

Fig 4. FTIR spectra of C. glutamicum ATTCC13032, graph-1 before bioreduction, graph-2 after bioreduction

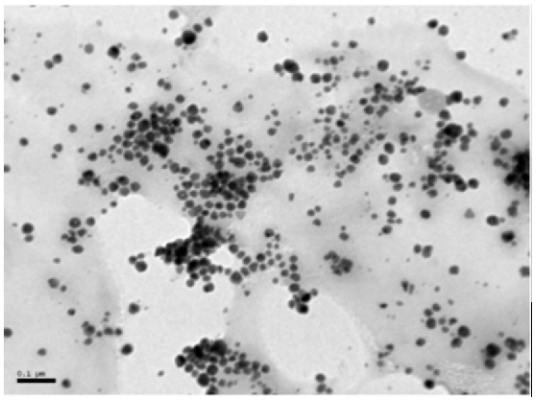

Fig 5a. GTEM image of silver nanoparticles high

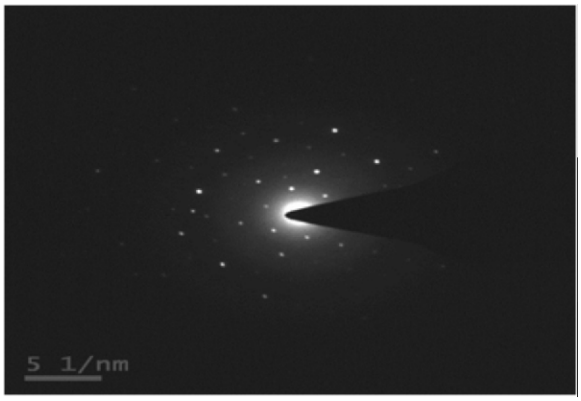

Fig 5b. Electron diffraction of AgNPs at resolution

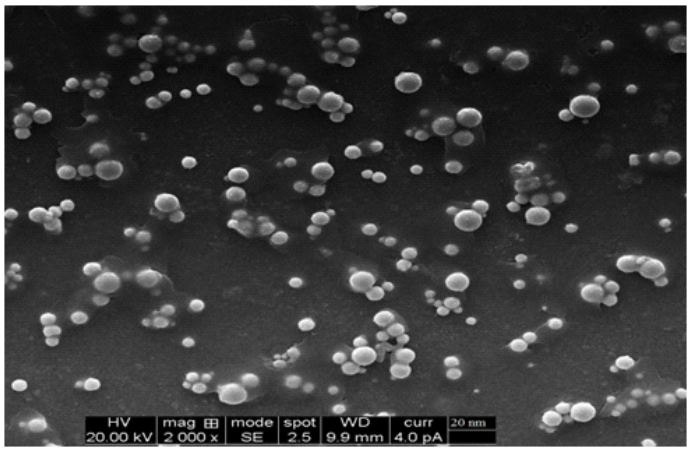

Fig 6. SEM image of synthesized AgNPs

cubic crystal structure of metallic silver. The high intense peak for FCC materials is generally (111) reflection, which is observed in the sample. The intensity of peaks reflected the high degree of crystallinity of the silver nanoparticles. However, the diffraction peaks are broad which indicating that the crystallite size is very small [33].

EDX studies

The elemental analysis of sample has been performed using energy dispersive spectroscopy (EDX) spectroscopy. The particles were checked and were found to contain a great deal of silver and the Fig. 8 shows EDX spectrum of silver nanoparticles. Strong peaks from the silver atoms in the nanoparticles are observed at 2.7, 3.0, 3.2, and 3.6 keV (Figure 8). The graph also shows the presence of zinc (Zn), potassium (K) and aurum (Au) in the EDX picture of silver nanoparticles. This is probably due to the presence of substrate over which the NP sample was held

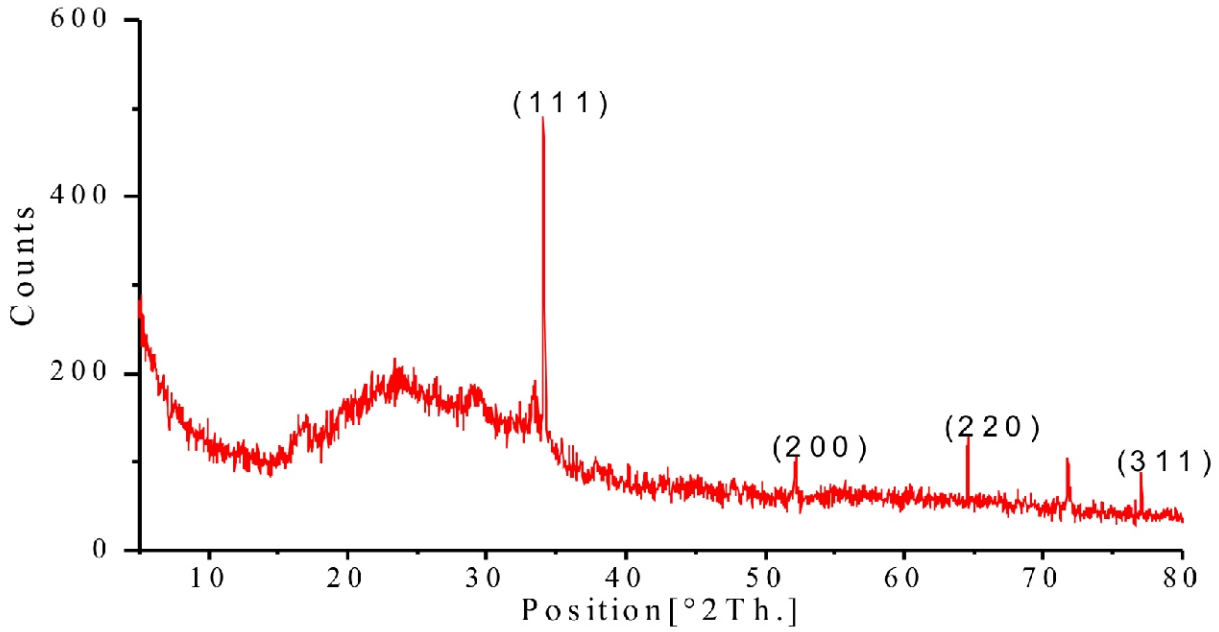

Fig 7. X-ray diffraction pattern of prepared silver nanoparticles

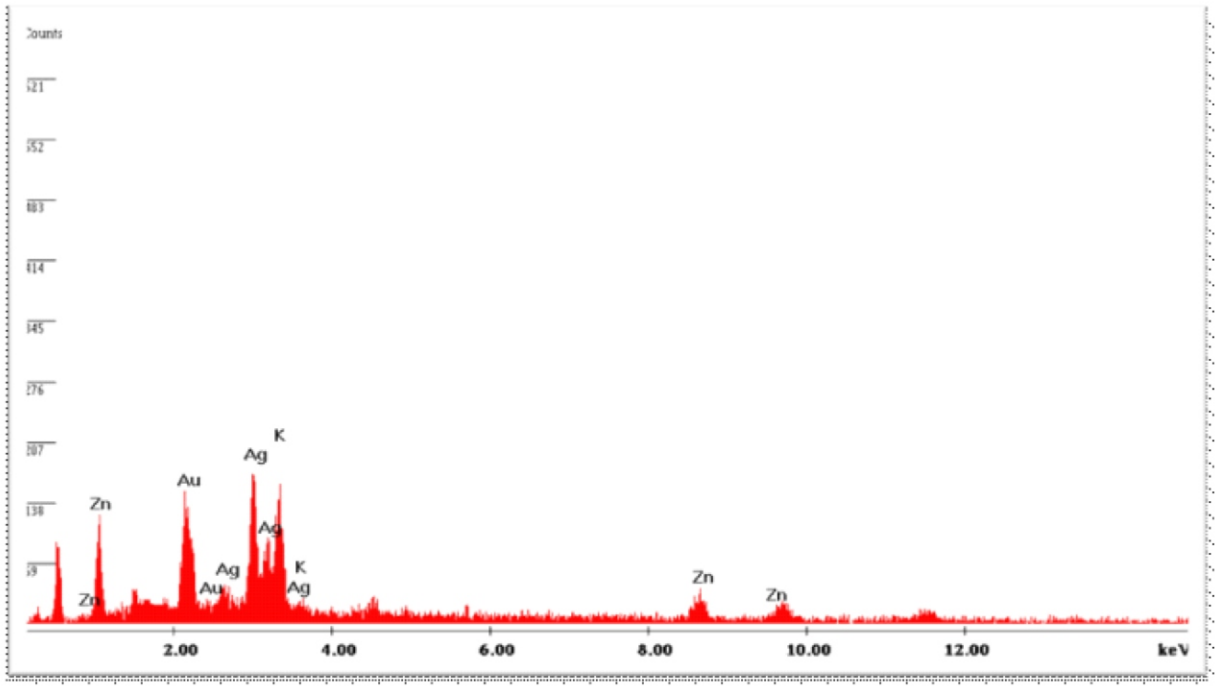

Fig 8. Energy-dispersive X-ray spectrum of nanoparticles

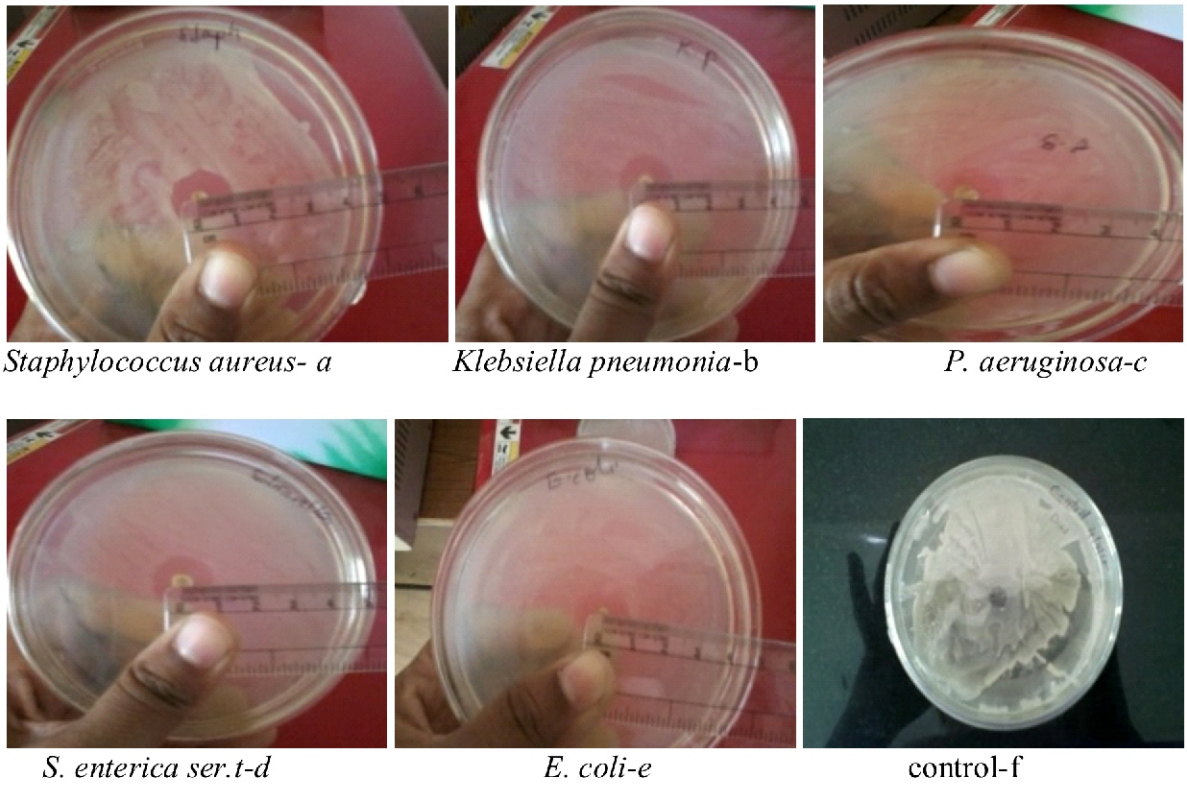

Fig 9. Antimicrobial activity of silver nanoparticles against bacterial pathogens used in the experiment

during SEM microscopy.

Antimicrobial activity

The antimicrobial efficiency of AgNPs against bacteria and fungi is useful in various medical applications. In the present study 0.025mg of the nanoparticles was taken as final product for antimicrobial assay. Antimicrobial activity of the synthesized silver nanoparticles was studied against bacteria (*S. aureus, Klebsiella pneumonia*, *S. enterica, Pseudomonas aueroginosa, E. coli)* Figure 9(a-e) and fungi (*Candida albicans, A.niger, A.flavus and Paeciliomyces variuti*) Figure 10 using well diffusion technique and the assay was performed in triplicate.

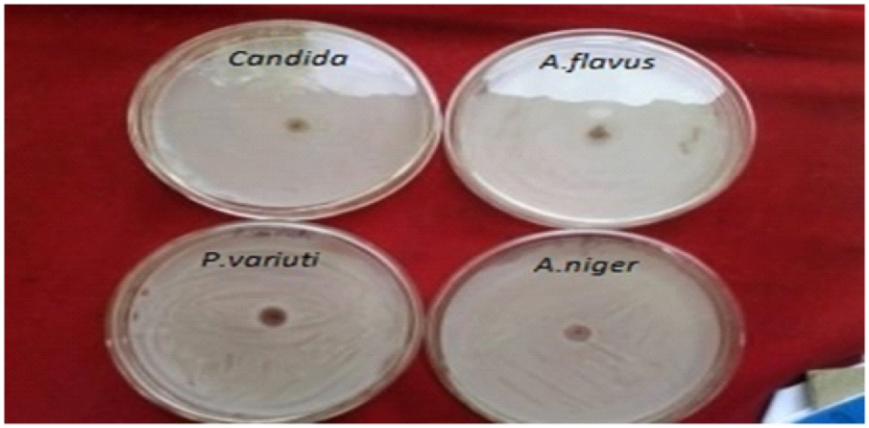

Fig 10. Antimicrobial activity of silver nanoparticles against fungi showing the inhibitory zone.

Control also maintained in which no zone of inhibition is observed (Figure 9f). From the mean values, highest antimicrobial activity was observed against bacteria than fungi. The maximum zone of inhibition was observed with *K.pneumonia* about 16mm in diameter. Whereas the cultures of *S.aureus*, *E.coli*, *S. enterica ser.t and P. aeruginosa* were also show zones of inhibition which was about 14mm, 12mm, 11mm, and 09mm in diameter respectively. Silver nanoparticles against the fungal cultures exhibited on the average of 3mm diameter zone of inhibition shown in Figure 10.

DISCUSSION

Synthesis of silver nanoparticles using biological method is easy and eco friendly. Formation of nanoparticles was identified by the change of color. The characteristics brown color of colloidal silver solution is due to the excitation of surface plasmon vibrations in the nanoparticle and provides a convenient spectroscopic signature of their formation [34]. Silver nanoparticles exhibit strong UV-Vis absorption spectra of electromagnetic waves in the visible range due to their optical resonant property, it occurs due to collective oscillation of conduction electrons, combined with the incident light [35]. The synthesized nanoparticles are showing strong absorption band between 410-440 nm; this is similar with the findings of Esumi et al., 2000 *and* Zhang et al., 2005 [36&17].

Nanoparticles formation was high at acedic pH range in particular at pH 2 and 5, this is due to some cell wall constituents of *C. glutamicum* ATTCC13032 might be hydrolyzed to a greater degree at the lower pH value, which hindered cell aggregation and partially degraded the bacterial cell wall. More active sites were thus exposed for biosorption [17]. But our results are contrasted with the results of Sneha et al., 2010 [37], where they got nanoparticle formation better at basic pH. The FTIR measurement also indicates that the aldehyde, amide and kentones present in the cell wall have the important role in reduction of $[Ag(NH_3)_2]^+$ to Ag^+ ions or nanoparticles. The positions of these bands are close to that reports of Zhang Haoran [17]. The size of synthesized silver nanoparticles ranged between 15-20nm. Our nanoparticles size is slightly contrasted with the nanoparticles of Zhang HR et al., and Sneha et al. [17& 37], where they got diameter ranged from 10-15nm sized nanoparticles. A report by Gurunathan et al., 2009a [38] showed that by controlling the environment of nanoparticle synthesis, silver nanoparticles of various sizes and shapes could be synthesized. At acidic pH the size of the nanoparticles ranged 45 nm whereas at pH 10 the size is just 15 nm. It is also due to the size of the particles increases with growth time [39]. Presence of silver nanoparticles was confirmed by XRD and EDX studies. The presence of optical absorbance band from EDX studies at ~3eV reveals the presence of pure metallic silver nanoparticles [40]. Silver nanoparticles produced from *C. glutamicum* strain were showing good antimicrobial activity. Many other studies [41-42] proved that AgNPs can be used as an antimicrobial agent. The exact mechanism which silver nanoparticles employ to cause antimicrobial effect is not clearly known. There are however various theories on the action of silver nanoparticles on microbes to cause the microbicidal effect. Silver nanoparticles have the ability to anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane like the permeability of the cell membrane and death of the cell. There is formation of pits on the cell surface, and there is accumulation of the nanoparticles on the cell surface [22]. The formation of free radicals by the silver nanoparticles may be considered to be another mechanism by which the cells die.

CONCLUSION

Silver nanoparticles were synthesized successfully from *C. glutamicum* strain which is generally regarded as non pathogen and easily growing organism. *C. glutamicum* strain is greatly reducing diamine silver into silver nanoparticles at acidic pH range; it is due to ionization of cell wall compounds mainly carboxyl and amide groups of proteins evidenced by FTIR spectra. Synthesized nanoparticles were showing Surface Plasmon Resonance (SPR) between 410-440nm. The TEM analysis indicates the presence of 10-20nm size of silver nanoparticles and XRD results further confirm the existence of AgNPs and the silver nanoparticles formed are crystalline. SEM images clearly evident that the synthesized silver nanoparticles were spherical in shape. Chemical analysis performed with the use of Energy Dispersive X-ray (EDX) revealed that the powder contains of silver. Antimicrobial potential of synthesized Ag nanoparticles was tested against infectious bacteria and fungi by well diffusion assay. The AgNPs was showing strong antimicrobial potential against bacteria than the fungi. Current study is eco-friendly and need cheaper cultivation requirements and higher growth rates on both industrial and laboratory scales, thereby having a lower cost in large-scale production of silver nanoparticles with good antimicrobial activity.

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Conflict of interest

The authors declare that they have no conflict of interest in the publication

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