SYNTHESIS AND CHARACTERIZATION OF SILVER NANOPARTICLES BY *RHIZOPUS STOLONIER*

Afreen Banu* and Vandana Rathod

Department of Microbiology Gulbarga University, Gulbarga -585106, Karnataka, India

E-mail of Corresponding author: afreen_sedam@yahoo.co.in

Abstract

Biological synthesis of silver nanoparticles by using fungi is reported here. The present study is on screening of filamentous fungi for the production of silver nanoparticles extracellularly. Eight fungi, *Rhizopus.spp, Aspergillus terreus, A.flavus, A.niger, A.clavatus, Acremonium.spp, A.rutilum, Trichoderma.sp.* have been screened for the production of silver nanoparticle. The fungal filtrates of the above said isolates were subjected to silver nitrate. After incubation, visual observation of brown color is an indication of silver nanoparticle production. Of the eight fungi, only one *Rhizopus stolonifer* showed maximum absorbance at 422nm. Parametric optimization study showed maximum absorbance at 40^{o}C and pH 7.0. Further characterization was made by UV-Visible absorption spectroscopy which shows maximum absorption at 422 nm, Transmission Electron Microscope (TEM) revealed the formation of spherical nanoparticles with size ranging between 5 to 50 nm. Energy Dispersive Spectroscope (EDS) shows the optical absorption peak at 3kev, Fourier Transform Infrared (FT-IR) shows the bands at 1645(1), 1537(2) and 1454(3) cm^{-1}.

Keywords: *R.stolonifer*, silver nanoparticles, FT-IR.

1. Introduction:

Silver nanoparticles are at the leading edge of the rapidly developing field of nanotechnology. Due to its unique properties, it is widely used in catalysis, chemical sensing and biosensing, photonics, electronics, optics and DNA sequencing, surface enhanced Raman spectroscopy and pharmaceuticals[1]. Nowadays, synthesis and characterization of nanoparticle is an important area of research[2]. Processes which are available presently for nanoparticle synthesis are chemical, physical and recently developed Biomimetics[3]. Some examples of physical methods are vapor deposition and lithographic processes, chemical methods include the popular borohydride and citrate

reduction methods for the preparation of colloidal metals (gold, silver etc) particles but these methods are being environmentally hazardous(chemical methods) and result in quick agglomeration of nanoparticles leading to big particles of poor monodispersity. Biomimetic is a technique in which we use biological systems such as bacteria, fungi, yeast and plants for synthesis of silver nanoparticles. It provides advancement over chemical and physical methods as it is cost effective and environment friendly. Therefore "Greener nano synthesis" has been proposed and preferred over these aforesaid chemical methods[4]. Of the different microbial sources fungi are known to secrete much higher amounts of proteins, thus have significantly higher productivity of nanoparticles. Mukherjee et al. (2001a,b) and Ahmed et al. (2003) reported gold and silver nanoparticles synthesis, extracellularly from *Verticillium sp* and *Fusarium oxysporum*.

Therefore, this study reports the extracellular synthesis of silver nanoparticles, using fungi isolated from the soil. Of the eight fungi, *Aspergillus terrus, A.flavus, A.niger, A.clavatus, Acremonium .sp, Rhizopus. spp, A.rutilum, Trichoderma .sp.* screened, *R. stolonifer* produced silver nanoparticles efficiently. Extracellular enzymes from the fungal filtrate and the aqueous solution of $AgNO_3$ were used for the production of silver nanoparticles. The use of specific enzymes such as reductases secreted by fungi opens up exciting possibilities of designing a rational biosynthetic strategy for metal nanoparticle of different chemical composition.

2. Materials and Method:

Eight fungal isolates from soil samples have been used in the study. Different fungal isolates were inoculated in Malt Glucose Yeast Peptone (MGYP) broth[5] containing yeast extract-0.3%, malt extract-0.3%, glucose-1%, peptone-0.5%, at 40^{o}c, in shaking condition (180 rpm). After the incubation the biomass was filtered and then extensively washed with distilled water to remove any medium component. This biomass was taken into sterilized flasks containing 100 ml distilled water. The flasks were incubated at the same condition as described above. The biomass was filtered again, (Whatman filter paper No.1) after the incubation period of 72 h the fungal filtrate was used further. Aqueous solution of $AgNO_3$ (1mM AgNO3 of final concentration) was mixed with fungal filtrate and the flasks were incubated on shaking condition. Periodically, aliquots of only those isolates which showed color change from

yellow to brown were subjected to UV-Visible absorption spectroscope TEM-EDS and FT-IR studies.

2.1. Optimization studies

There is always an interaction continuously between the organism and the environment they live in. The environmental conditions exert an influence which will have meaningful bearing on the growth, development and very existence of organisms. For an organism to succeed, it must live in balance with its surrounding environment[6]. The enzyme production by fungi is influenced by the condition in which the organisms are cultivated[7]. Therefore, parametric optimization studies will have greater influence on the enzyme production, which will influence silver nanoparticle production.

2.2. Effect of Physical Parameters

The physical factors have been the critical components of an industrial and also the process economics[8]. Optimization of physical parameters will not only support good growth but also enhance product yield. The effect of various temperatures ranging from 25°C to 45°C (with a difference of 5°C) on *R. stolonifer* were studied for silver nanoparticle production. Different pH values ranging from 4.0 to 8.0 (with a difference of 1.0) were used to study the influence of pH on nanoparticle production.

2.3. Characterization of Silver Nanoparticles

Absorption spectroscopy in the UV-Visible region has long been an important tool for the nanoparticle characterization. UV-Visible absorption spectrophotometer (T90/T90+ doublebeam) with a resolution of 1 nm was used for recording the absorption spectra, which is one of the important technique to verify the formation of metal nanoparticles provided surface plasmon resonance exists for the metal [12]. The presence of elemental silver was confirmed through energy dispersed spectroscope. Transmission electron micrograph pattern were recorded on a carbon coated copper grid on a Hitachi-H-7500 machine. The interaction between protein and AgNPs was analysed by fourier transform infrared spectroscope (JASCO FT/IR-3500).

3. Results and Discussion:

Eight molds *Rhizopus.spp, Aspergillus terreus, A.flavus, A.niger, A.clavatus, Acremonium.spp, A.rutilum, Trichoderma.spp* were screened for their ability to synthesize silver nanoparticles from $AgNO_3$. Of them, only *R. stolonifer.* (Fig.1) showed brown color. Hence, further work was carried out with only this

fungus. The fungal biomass after the incubation period of 72 h was separated by filtration, which showed yellow color and gives brown color on completion of the reaction with Ag ions within 24h. The appearance of brown color solution (Fig-2) clearly indicates the formation of silver nanoparticles[9]. The color change was caused by the surface plasmon resonance (SPR) of Ag nanocrystals in the visible region (Varshney et al. 2009). Silver nanocrystals are known to exhibit size- and shape-dependent SPR bands which are characterized by UV–Visible absorption spectroscopy[10]. This event clearly indicates that the reduction of the ions occur extracellularly through reducing agents released into the solution by fungi. The UV–Visible absorption spectroscopy of the *R. stolonifer* at different times of the reaction is presented in (Fig-2).

3.1. The Effect of pH

The effect of varying pH on the biosynthesis of AgNPs by *R.stolonifer* is depicted in figure-3. It can be clearly seen from the UV-Visible absorption spectra that the absorption maxima shows the sharp peak at pH 7.0. But at pH 4 the broadening of absorbance is observed for AgNPs solution indicating the aggregation of particles. This observation can be attributed to the capping proteins secreted by the fungus in the solution are very much stable at high pH and the capping protein retains its characteristics as can be seen from FT-IR measurement (figure-4)and hence the AgNPs in solution remains stable at pH 7. But at low pH the protein structure gets affected and the protein gets denatured and loses its activity, thus aggregation of nanoparticles is observed. Thus, it can be concluded from above observation that the protein secreted by *R.stolonifer* in the solution for the capping of AgNPs are stable at alkaline pH but not at acidic pH which can be attributed to the stability of capping proteins.

3.2. The Effect of Temperature

The effect of varying temperatures on silver nanoparticle production by *R.stolonifer* is presented here. Study temperature range is from 25^{o}C to 45^{o}C maximum production was attained at 40^{o}C. Temperature is an essential factor affecting silver nanoparticle production. Our present data indicates that the optimum temperature of 40^{o}C is rather specific for silver nanoparticles production by *R.stolonifer*. (figure -5) which shows maximum absorbance at 422nm.

Parametric optimization studies revealed that temperature of 40^{o}C and pH 7.0 was favorable for the production of silver nanoparticles by *R.stolonifer*.

3.3. Characterization of silver nanoparticles

UV-Visible absorption spectroscopy is one of the most widely used techniques for structural characterization of silver nanoparticles. In our experiment the maximum absorbance was observed at 422nm, implying that the bioreduction of the silver nitrate has taken place following incubation of the $AgNO_3$ solution in the presence of cell-free extract. Our results are correlating with the reports of Sadowski, et al, (2008) and Maliszwaska, et.al., (2009) with the fungus *Penicillium*. Surface plasmon peak was located at 420 nm using *klebsiella pneumonia* (Minaeian et al 2008). Mukherjee et al, (2007) reported an intense peak at 410nm. It is reported that the absorption spectrum of spherical silver nanoparticles presents a maximum between 420nm and 450nm (Maliszewska 2008).

A representative TEM image recorded from drop coated film of a silver nanoparticles are spherical in shape. All the particles are well separated and no agglomeration was noticed. The size ranges between 5nm to30 nm was seen. The process of growing silver nanoparticles comprises of two key steps: (a) bioreduction of $AgNO_3$ to produced silver nanoparticles and (b) stabilization and/or encapsulation of the same by suitable capping agents[11]. It is suggest that the biological molecules could possibly perform the function for the stabilization of the AgNPs.

EDS analysis gives the additional evidence for the reduction of silver nanoparticles to elemental silver. The optical absorption peak is observed approximately at 3kev, which is typical for the absorption of metallic silver nanocrystals due to surface plasmon resonsnce, which confirms the presence of nanocrystalline elemental silver. Spectrum shows strong silver signal along with weak oxygen and carbon peak, which may be originate from the biomolecules that are bound to the surface of nanosilver particles.

The aim of IR spectroscopic analysis is to determine chemical functional groups in the sample. 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. Different functional groups absorb characteristic frequencies of IR radiation. Thus, IR spectroscope is an important and popular tool for structural elucidation and compound identification. The FTIR spectrum of the SNPs produced by *R.stolonifer* is shown in figure 4. This spectrum shows the presence of band at

1645(1), 1537(2) and 1454(3) cm-1, the bands at 1645 cm-1 corresponds to primary amine NH band.[12] The band at ca.1454 cm-1 due to methylene scissoring vibrations present in the proteins. Overall the observation confirms the presence of protein in the sample which coat covering the silver nanoparticles known as capping proteins. Capping protein stabilizes the metallic nanoparticle and prevents agglomeration in the medium. This study gives the evidence of formation and stabilization of silver nanoparticles in the aqueous medium by using biological molecules.

4. Conclusion:

The use of fungi in the synthesis of nanoparticle is a relatively recent addition to the list of microorganisms. The shift from bacteria to fungi as a means of developing natural "nanofactories" has the added advantage that downstream processing and handling of the biomass would be much simpler. The use of eukaryotes is potentially exiting since they secrete large amount of proteins, thus increasing productivity, and their easy usage in laboratory works is a suitable option in production of metallic nanoparticles among other microorganisms. More over the process can be easily scaled up, economically viable with the possibility of easily covering large surface areas by suitable growth of mycelia. Therefore, the present study has reported the biological process for the synthesis of silver nanoparticles extracellularly using *R.stolonifer*. *Rhizopus stolonifer* showed maximum absorbance at 422nm. Parametric optimization study showed maximum absorbance at 40^{o}C and pH 7.0. Further characterization was made by UV-Visible absorption spectroscopy which shows maximum absorption at 422 nm, Transmission Electron Microscope (TEM) revealed the formation of spherical nanoparticles with size ranging between 5 to 50 nm. Energy Dispersive Spectroscope (EDS) shows the optical absorption peak at 3kev, Fourier Transform Infrared (FT-IR) shows the bands at 1645(1), 1537(2) and 1454(3) cm-1. The biosynthesized silver nanoparticles have broad range of applications such as fluorescent biological labels, drug and gene delivery, bio detection of pathogens, detection of proteins, probing of DNA structure, tissue engineering, tumor destruction via heating, separation and purification of biological molecules and cells, and phagokinetic studies. Majority of the silver nanoparticle applications in medicine are geared towards drug delivery. Therefore, further work will be on antibacterial activity of silver nanoparticles.

Bibliography:

1. Varshney,R A.N. Mishra, S.Bhadauria, M.S.Gaur , 2009. A novel microbial route to synthesize silver nanoparticles using fungi Hormoconis resinae. Digest journal of Nanomaterials and Biostructures Vol. 4, No.2, p.349-355.
2. Mritunjai Singh, Shinjinin Singh, S.Prasad, I.S.Gambhir, 2008. Nanotechnology in medicine and antimicrobial effects of silver nanoparticles. Digest Journal of Nanomaterials and Biostructures vol.3, No.3, P.115-122.
3. Upendra kumar Parashar , Preeti S. Saxena, Anchal Srivastava, 2009. Digest journal of Nanomaterials and Biostructures vol.4,No.1,March 2009,p. 159-166.
4. Mukherjee P, M Roy, B P Mandal, G K Dey, P K Mukherjee, J Ghatak, A K Tyagi and S P Kale,2008. Green synthesis of highly Stabilized nanocrystalline silver particles by a non- pathogenic and agriculturally important fungus T.asperellum. IOP publishing Nanotechnology journal 19: 075103(7pp).
5. Karbasian M, Atyabi SM, Siyadat SD, Momen SB and Norouzian D, 2008.Optimizing Nano-silver Formation by Fusarium oxysporum PTCC 5115 Employing Response Surface methodology. American journal of Agricultural and biological science 3(1): 433-437.
6. Rose, A.H. (1989). Influence of the environment on microbial composition, In-Microbial Lipids, Ratlidge, C. and Wilkinson, S.G.(ed.) Vol.2.Harcourt, Brace Jovanovich, Publishers. Academic press, New York, P. 255-278.
7. Lekha, P.K. and Lonsane, b.K. (1997). Production and application of tannin acyle hydrolase: State of the art.Adv.Appl.Microbiol., 44:215-260.
8. Zhang, and Greasham,R. (1999). Chemically defined media for commercial fermentations. Appl. Microbiol. Biotechnol., 51:407-421.
9. Satyajoti Senapati , (2005). Biosynthesis and immobilization of Nanoparticles and their applications,PhD thesis.
10. Jianping Xie,† Jim Yang Lee,†,§,* Daniel I. C. Wang,†,‡ and Yen Peng Ting§; American chemical society , Vol. 1 ▪ No. 5 ▪ 429–439 ▪ 2007.
11. P.Mukherjee, M Roy, B P Mandal, Green synthesis of highly stabilized

nanocrystaline silver particles by a non-pathogenic and agriculturally important fungus T. asperellum. Nanotechnology 2008; 19, 075103(7pp).

12. Amanulla Mohammed Fayaz, Journal of Nanomedicine: NBM. 6 (2010) 103-109

Figure-1: *Rhizopus sp* on PDA plate produced

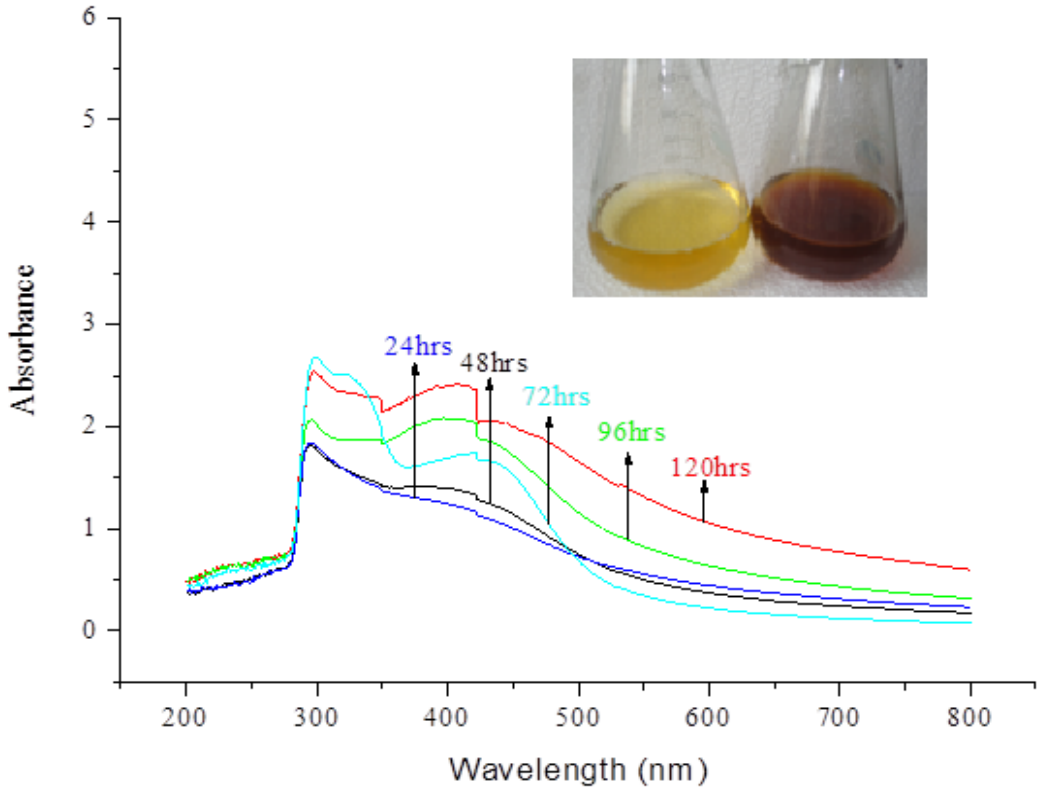


Figure 2: UV-Visible absorption spectra of AgNPs by *R.stolonifer*

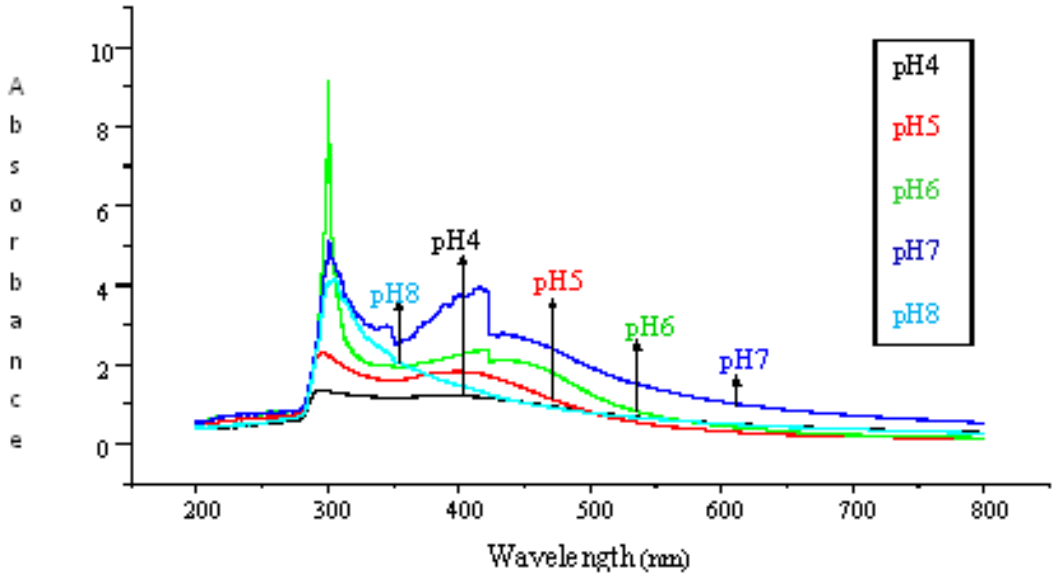
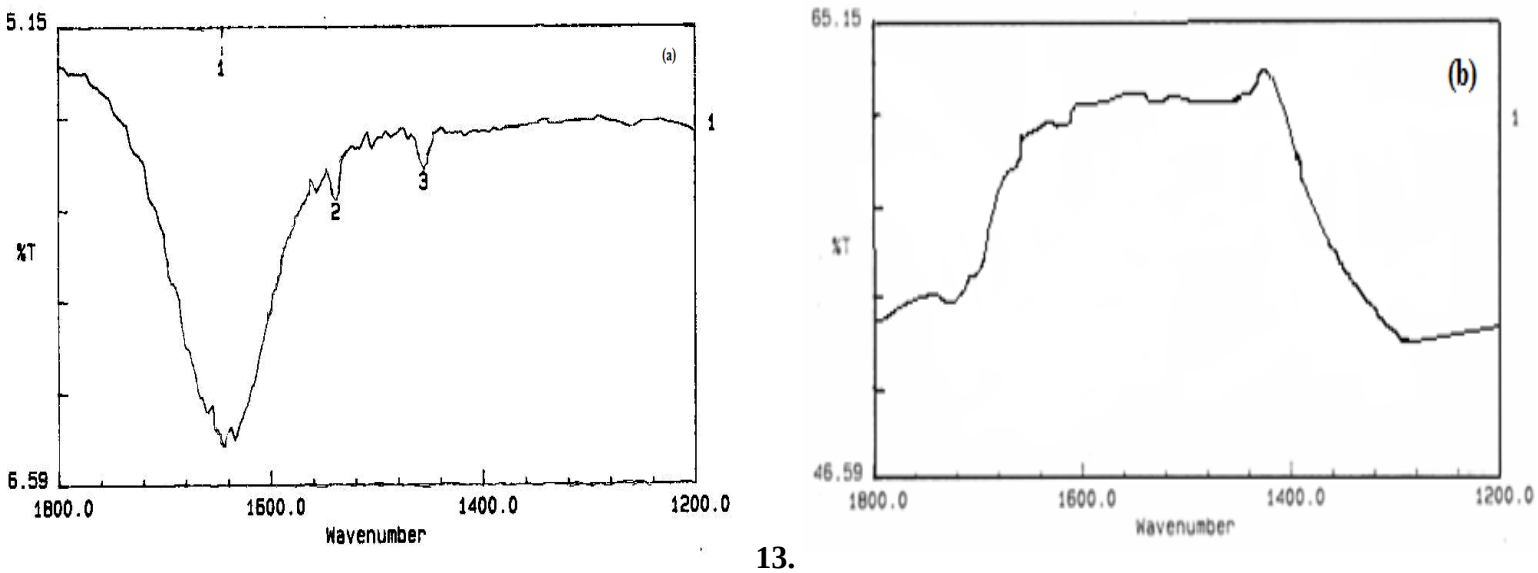


Figure 3: The effect of pH on production of nanosilver by *R.stolonifer*



13.

Figure 4: FT-IR spectra recorded from a drop-coated film of nanoparticles-fungus reaction mixture after 48 h of reaction (a) at pH 7 (b) at pH 4.

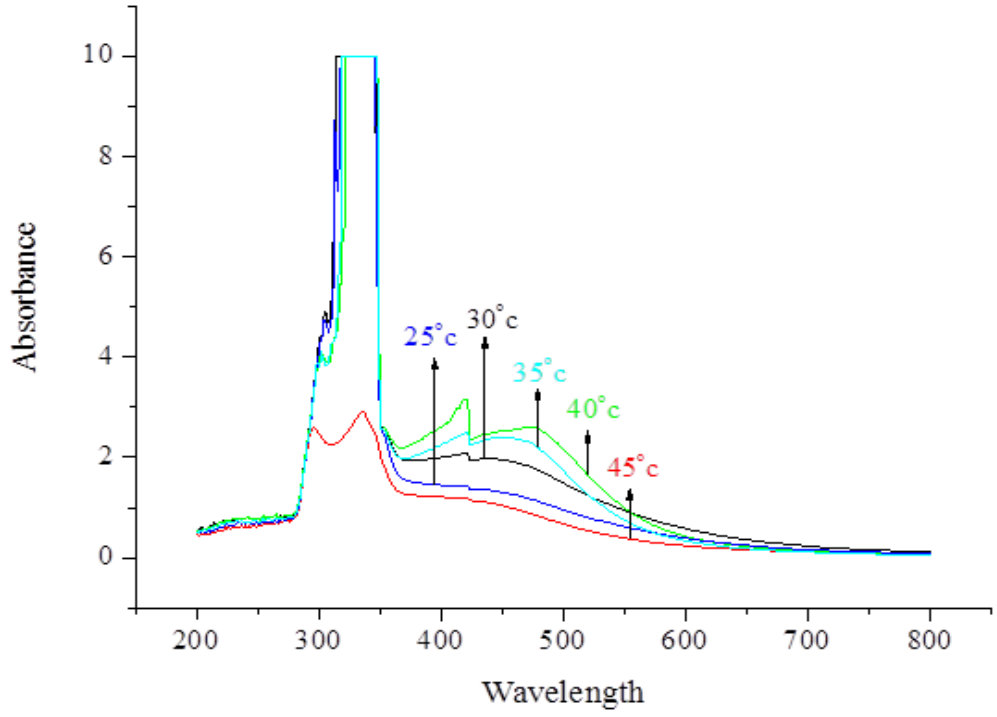


Figure 5: The effect of temperature on production of nanosilver by *R.stolonifer*

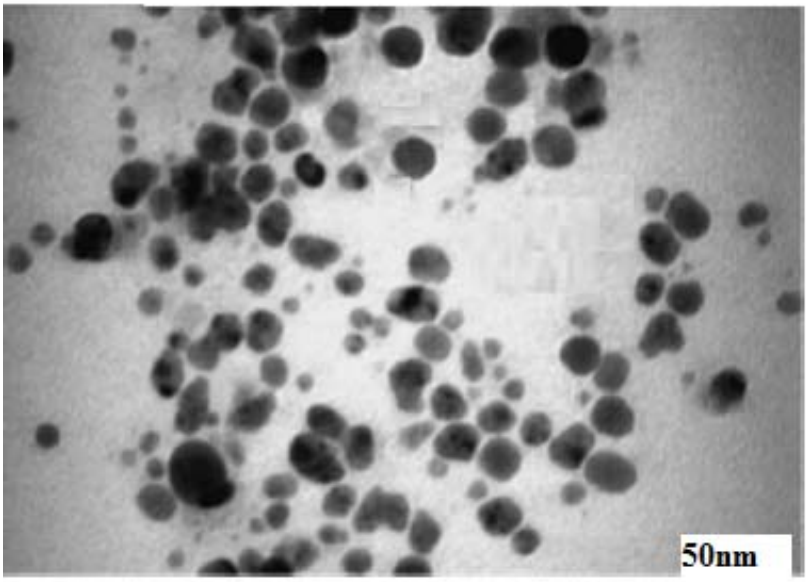


Figure 6: TEM image of nanosilver produced by *R.stolonifer*

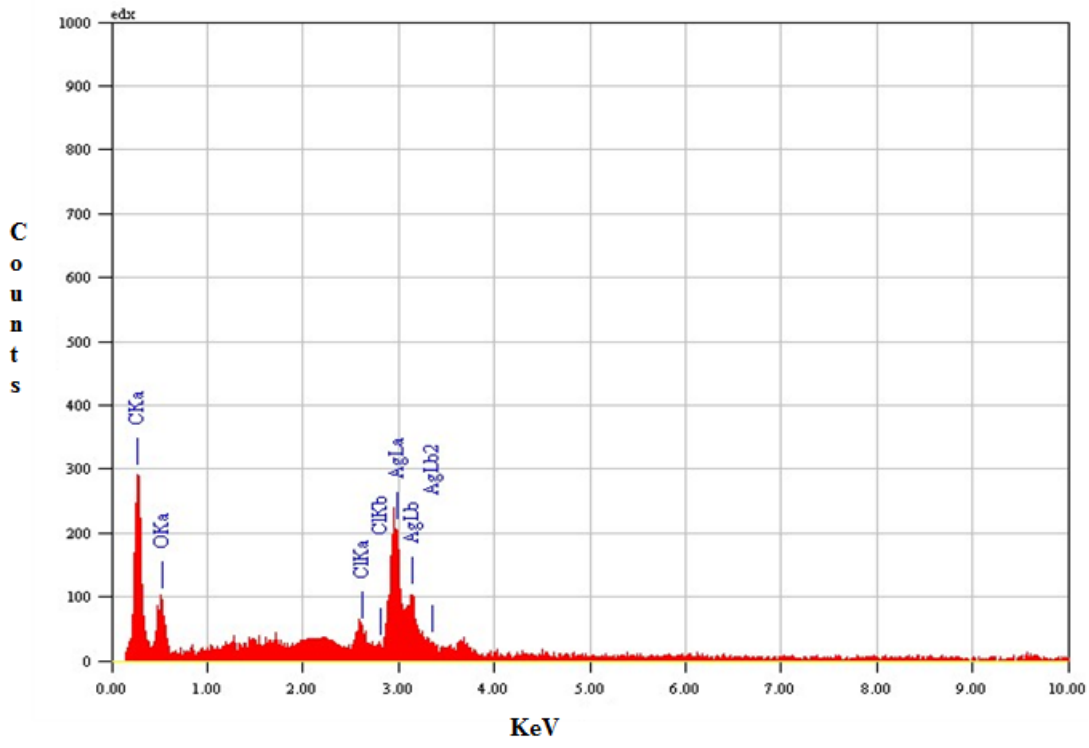


Figure 7: EDS of biosynthesized silver nanoparticles