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Biosynthesis, optimization, purification and characterization of gold nanoparticles

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Many microorganisms produce intracellular metal nanoparticles. Aqueous gold, when exposed to several actinomycetal strains, become thereby, leading to the formation of gold nanoparticles. The use of microorganisms in the synthesis of nanoparticles is emerging as an eco-friendly and exciting approach gold for recovery. *Streptomyces hygroscopicus* was used for the biosynthesis of gold nanoparticles. UV and visible spectroscopic studies of biofilms revealed better synthesis of nanoparticles. It was observed that better biosynthesis of gold nanoparticles occurred when cell biomass treated with 10^{-3} and 10^{-4} mM $HAuCl_4$ solution as compared to other dilutions. The pH 7.0 was found to be optimum for the biosynthesis of gold nanoparticles. The TEM study revealS that there was evidence of gold nanoparticles synthesized by *S. hygroscopicus*. The results demonstrate that spherical gold nanoparticles in the range of 10 to 20 nm were observed at pH value of 7.0. The actinomycetal biomass and various concentration of aqueous $HAuCl_4$ solution were incubated, it was found that 10^{-4} concentration shows excellent colour of the actinomycetal biomass.

Key words: Biosynthesis, optimization, gold nanoparticles, purification, characterization.

INTRODUCTION

Chemical production processes for metal nanoparticles are not regarded as being environmentally friendly (Sastry et al., 2003; Gamez et al., 2002) and generally yield only spherical nanoparticles. On the other hand, a wide variety of geometric, metal nanoparticles were produced by both prokaryotic and eukaryotic organisms including bacteria, fungi and yeasts (Sastry et al., 2003; Ahmad et al., 2003; Mukherjee, 2001). This bioreduction of metal particles is regarded as an organism's survival mechanism against toxic metal ions and occurs *via* an active or passive process or a combination of the two (Duran et al., 2005; Ibrahim et al., 2001). Such a biological route involving micro-organisms provides great advantages over traditional methods, as it has the potential to be cost-effective, simple and environmentally friendly. The primary advantage of biological route is the ability, in theory to manipulate the properties of the nanoparticles by gaining control over the mechanism that determines their size and shape (Ahmad et al., 2003; Mukherjee, 2001). Many microorganisms, both unicellular and multicellular are known to produce inorganic materials either intracellularly or extracellularly (Mann, 1996; Lloyd, 2003) often of nanoscale dimensions and of exquisite morphology and hierarchical assembly. Some well-known examples of microorganisms synthesizing inorganic materials include magnetotactic bacteria (which synthesize magnetite nanoparticles) (Ahmad et al., 2003) and actinomycetes such as the extremophilic actino-

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mycete; *Thermomonospora* sp. (Ahmad et al., 2003) and the alkalotolerant actinomycete *Rhodococcus* sp. (Fortin and Beveridge, 2000). Both live microorganisms and dead microorganisms are gaining importance by virtue of their facile assembly of nanoparticles. Gold particles of nanoscale dimensions may be readily precipitated within bacterial cells by incubation of the cells with Au^{3+} ions (Klaus et al., 2001).

The bacterium *Pseudomonas stutzeri* AG259 isolated from a silver mine, when placed in a concentrated aqueous solution of $AgNO_3$, led to the reduction of the Ag+ ions and to the formation of silver nanoparticles of well-defined size and distinct morphology within the periplasmic space of the bacteria (Mukherjee et al., 2001). Eukaryotic organisms such as fungi may be used to grow nanoparticles of different chemical compositions and sizes. A number of different genera of fungi have been investigated in this regard and it has been shown that fungi are extremely good candidates in the synthesis of gold (Mandal et al., 2006; Mukherjee et al., 2001) or silver (Ahmad et al., 2003; Duran et al., 2005; Gardea-Torresdey et al., 2002; Varshney et al., 2009) particles. Several attempts of synthesis of metal nanoparticles have been made by researchers. Very recently, it was also shown that *Fusarium oxysporum* produced optoelectronic material Bi_2O_3 nanocrystals in the size between 5 to 8 nm extracellularly with quasispherical morphology and good tunable properties. When bismuth nitrate was added as precursor, the as-synthesized nanocrystals were in monoclinic and tetragonal phases (Uddin et al., 2008). *F. oxysporum* (Mukherjee et al., 2002), *Colletotrichum* sp. (Shankar et al., 2003) and *Trichothecium* sp. (Ahmad et al., 2005) produced extracellular gold nanoparticles with spherical, triangular and hexagonal morphologies with 5 to 200 nm in size. It was also intriguing to observe that the silver nanoparticles produced by *T. asperellum* (Mukherjee et al. 2008), *T. viride* (Fayaz et al., 2010), *F. oxysporum* (Senapati et al., 2004), *P. chrysosporium* (Vigneshwaran et al., 2006), *F. solani* (Ingle et al., 2009), *F. semitectum* (Basavaraja et al., 2008), *F. acuminatum* (Shankar et al., 2003), *A. fumigatus* (Bhainsa and D'Souza, 2006), *C. versicolor* (Sanghi and Verma, 2009), *A. niger* (Gade et al., 2008), *P. glomerata* (Birla et al., 2009), *P. brevicompactum* (Shaligram et al., 2009), *C. cladosporioides* (Balaji et al., 2009), *P. fellutanum* (Kathiresan et al., 2009) and *V. volvacea* (Philip, 2009) were predominantly spherical with pyramidal, rod-like and triangular morphologies in the size of 5 to 200 nm.

The purification of water-soluble gold nanoparticles is particularly difficult because the nanoparticles and the impurities have similar solubility, often making standard purification techniques (that is, precipitation, extraction, chromatography, centrifugation or dialysis) inadequate or inefficient (Brust et al., 1995; Kanaras et al., 2002; Weare et al., 2000; Brust et al., 1994). Effective purification of nanoparticles is, therefore, a necessary step for controlling the quality and characteristics of nanoparticle products (Dalwadi et al., 2005).

MATERIALS AND METHODS

Actinomycetal isolate

The *S. hygroscopicus* metal tolerant actinomyctal isolate was obtained from soil samples of Eastern Balaghat ranges of Maharashtra, India.

Preparation of biomass

S. hydroscopicus was grown in 500 ml Erlejnmeyer flask containing 100 ml of sterile Malt Extract Glucose Yeast Extract Peptone (MGYP) both supplemented with griseofulvin at 50 µg/ml. Incubation was with shaking (200 rpm) at 35°C for 4 days. The flasks were removed from the shaker and placed at 5 to 10°C, to let the mycelium settle. The supernatant fluid was discarded and 100 ml of sterile distilled water was added for washing the cells. The flasks were kept at 5 to 10°C for 30 min to let the mycelium settle again. The supernatant fluid was poured off slowly to discard. 100 ml sterile distilled water was again added to the flask, and this procedure was repeated three more times. The mycelial mass was then seperated from the sterile distilled water by centrifugation (1500 rpm) for 10 min; the mycelial pellets were weighed and used for the synthesis of gold nanoparticles.

Preparation of metal stock solutions

333.79 g of $HAuCl_4$ in 1000 ml of distilled water were used to obtain the 10 M.

Exposure of biomass to metal solutions

Five grams of actinomycetal wet biomass were exposed to 50 ml of a sterilezed aqueous solution of $HAuCl_4$ at varying concentrations in 250 ml Erlenmeyer flasks and the flasks placed on a shaker at 200 rpm and incubated at 35°C for 4 days.

Characterization of metal nanoparticles

Visual observations

Samples of the reaction mixtures were verified visually for a possible colour change after 12, 24, 48 and 72 h of incubation (Table 1). The change in colour from pale yellow to a pinkish appearance was indicative of the formation of gold nanoparticles.

U. V. and visible spectroscopy

Biosynthesis of metal ions was also monitored by taking 2 ml aliquots of reaction mixture at different time intervals and centrifuging them at 5000 rpm for 10 min. The centrifuged biomass was washed twice with double distilled water and biofilms were prepared. The biofilms were dried in an oven at 45°C for 1 h and examined by spectroscopic analysis using an SL 159 U. V. and visible spectrophotometer (300 to 800 nm). The biomass samples showing the desired color change were used for further studies.

Preparation of samples for SEM

The presence of nanoparticles was confirmed by SEM. The metal

Table 1. Visual observation on *S. hygroscopicus* biomass colour after treatment with various $HAuCl_4$ solutions.

Molarity	Colour of biomass (control)	Colour change in biomass			
		After 12 h	After 24 h	After 48 h	After 72 h
10^{-1}	Yellowish white	Yellowish white	Yellowish white	Yellowish white	Yellowish white
10^{-2}	Yellowish white	Yellowish white	Yellowish white	Yellowish white	Faint pink
10^{-3}	Yellowish white	Faint pink	Purple	Purple	Violet
10^{-4}	Yellowish white	Yellowish white	Yellowish white	Yellowish white	Faint pink
10^{-5}	Yellowish white	Yellowish white	Yellowish white	Yellowish white	Yellowish white

treated biomass was centrifuged at 1500 rpm for 20 min and washed twice with double distilled water. The washed biomass was sonicated and homogenized using homogenizer. The homogenate was applied onto glass slide as an uniform thin layer and fixed in 2.5% glutaraldehyde for 12 h at room temperature. After fixation, the sample was subjected to dehydration in increasing concentrations of alcohol namely, 25, 50, 75 and 100% for 15 min each time. After platinum sputtering coating samples were examined by SEM.

Scanning electron microscopy (SEM)

The prepared biofilms were mounted onto carbon-coated copper grid. Micrographs were obtained using a JEOL (6360) JED-2300 analysis station operating at 200 kV.

Transmission electron microscopy (TEM)

The biomass samples were dispersed in water, left for 5 min in an ultra sonicator and, then, left to rest for 10 min. One drop of suspension was placed onto a grid of copper coated with 300 mesh palladium and carbon. Grids were examined using Zeiss CEM902 microscope at 80 kV.

Optimization of the biosynthesis of gold and silver nanoparticles

Effect of metal concentration

10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} mM solutions of $HAuCl_4$ were prepared 50 ml volumes of each solution were transferred into 250 ml Erlenmeyer flasks. The metal solutions were sterilized at 121°C for 30 min. The freshly prepared and washed *S. hygroscopicus* biomass with solution without incubation was kept in refrigerator to serve as control.

Two gram of freshly prepared and washed biomass were added to each flask and the flask incubated at 35°C (150 rpm) for 72 h. Every 12 h, a 2 ml sample was collected, centrifuged at 1500 rpm at for 20 min, the biomass (pellet) recovered, biofilms prepared, and absorbance biofilm was determined at 550 nm with the help of U. V. visible spectrophotometer.

Effect of pH

50 ml volumes of the $HAuCl_4$ (10^{-4} mM) solution were placed into seven 250 ml Erlenmeyer flasks. pH of the metal solution was adjusted with 0.1 N NaOH to 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, respectively. The metal solution was sterilized at 121°C for 30 min. The freshly prepared, washed *S. hygroscopicus* cell biomass with metal solution without incubation was kept at refrigerator as a control.

2 g of freshly prepared and washed biomass were added to each one of the aforementioned seven flasks and the flasks were incubated at 35°C (150 rpm) for 72 h. After 12, 24, 48 and 72 h of incubation, a 2 ml sample was collected from each flask, centrifuged at 1500 rpm at 10°C for 20 min and the biomass (pellet) recovered, biofilms were prepared as described earlier and absorbance was determined at 550 nm with the help of U. V. visible spectrophotometer.

Partial purification of gold nanoparticles

The HAuCl4 treated and washed biomass samples containing nanoparticles were collected, placed in crucibles and sequentially subjected to drying at room temperature for 24 h, at 45°C for 1 h, at 60°C for 1 h and, lastly at 200°C overnight. The ashes so obtained contained partially purified gold nanoparticles.

RESULTS AND DISCUSSIONS

It was found that the yellowish colour of *S. hygroscopicus* biomass when exposed to various $HAuCl_4$ solutions changed gradually (Table 1). A dark violet colour appeared over time under some conditions (Figure 1). The formation of such a violet colour is indicative of the intracellular formation of gold nanoparticles. It was also observed that after 72 h treatment, the aqueous $HAuCl_4$ solution was colorless, thereby indicating that the extracellular reduction of the $HAuCl_4$ ions has not occurred. The significant colour change after 72 h was observed only with the 10^{-4} mM solution.

Effect of $HAuCl_4$ metal at various concentrations on biosynthesis of nanoparticles

The effect of $HAuCl_4$ concentration on synthesis of gold nanoparticles by *S. hygroscopicus* was studied by U. V. visible spectroscopy. Absorbance of $HAuCl_4$ treated biofilm after 72h exposure at concentration of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} mM was determined at different wavelength and given in Figure 2. It was observed that maximum absorption at critical wavelength (550 nm) was obtained at 10^{-3} and 10^{-4} mM $HAuCl_4$ concentration as compared to 10^{-1}, 10^{-2} and 10^{-5} mM $HAuCl_4$ concentrations.

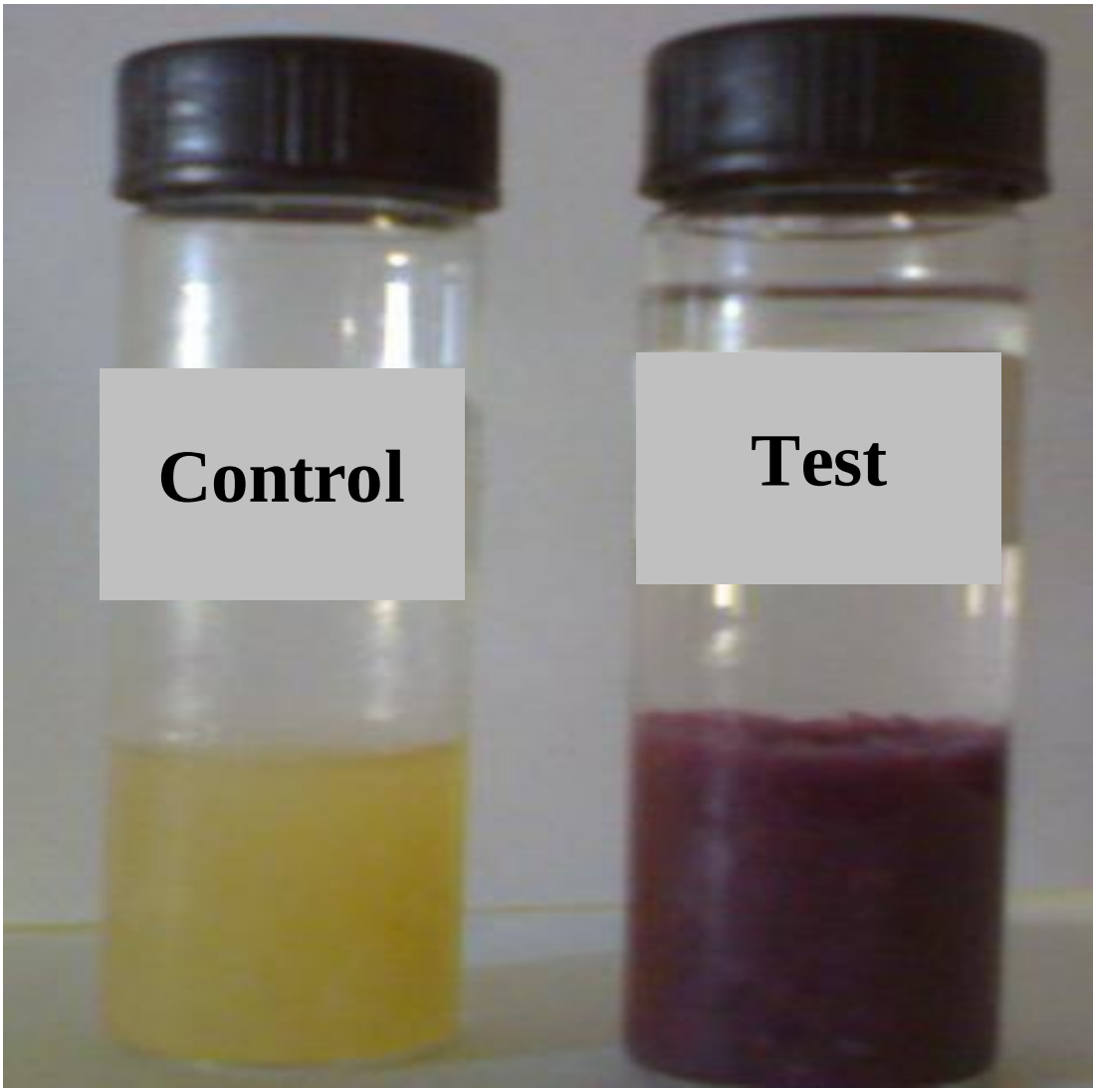

Figure 1. *S. hygroscopicus* biomass (control) before exposure to the 10^{-4} mM aqueous $HAuCl_4$ solution, (test) after exposure to 10^{-4} mM aqueous solution $HAuCl_4$ for 72 h.

Effect of pH on biosynthesis of nanoparticles

The effect of pH on synthesis of gold nanoparticles by actinomycetes was studied using U. V. visible spectroscopy. *S. hygroscopicus* was exposed for 72 h to 10^{-4} mM $HAuCl_4$ solution adjusted at various pH namely, 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. At the end absorbance was determined at different wavelength and given in Figure 2. It was found that optimum pH for the biosynthesis of $HAuCl_4$ nanoparticles was 7.0 (Figure 3). The pH was found to be an important parameter affecting gold nanoparticle synthesis. Variations in pH during exposure to Au-ions had an impact on the size, shape and number of particles produced per cell (Gericke and Anthony, 2006; Suryawanshi and Deshmukh, 2008; Kathiresan et al., 2009). The optimum gold accumulation by microbial cells normally occurs in the pH range of 2 to 6 (Joerger and Klaus, 2000) and changes in the pH has an effect on the size of gold nanoparticles (Mukherjee et al., 2001). *Lactobacillus* sp. showed that changes in the pH could have an effect on the size distribution of gold nanoparticles (Nair and Pradeep, 2002). Typical bright field transmission electron microscopy of aqueous 10^{-4} $HAuCl_4$ treated *S. hygroscopicus* biomass before and after 72 h exposure are given in Figure 4. The TEM image clearly shows presence of well-separated and almost spherical-shaped metal nanoparticles in the biomass after 72 h exposure; whereas, aggregate of metals are seen in the metal treated biomass at 0 h of exposure. Biosynthesized gold nanoparticles by *S. hygroscopicus* were partial purified and characterized by SEM. It was found that gold nanoparticles were almost spherical in

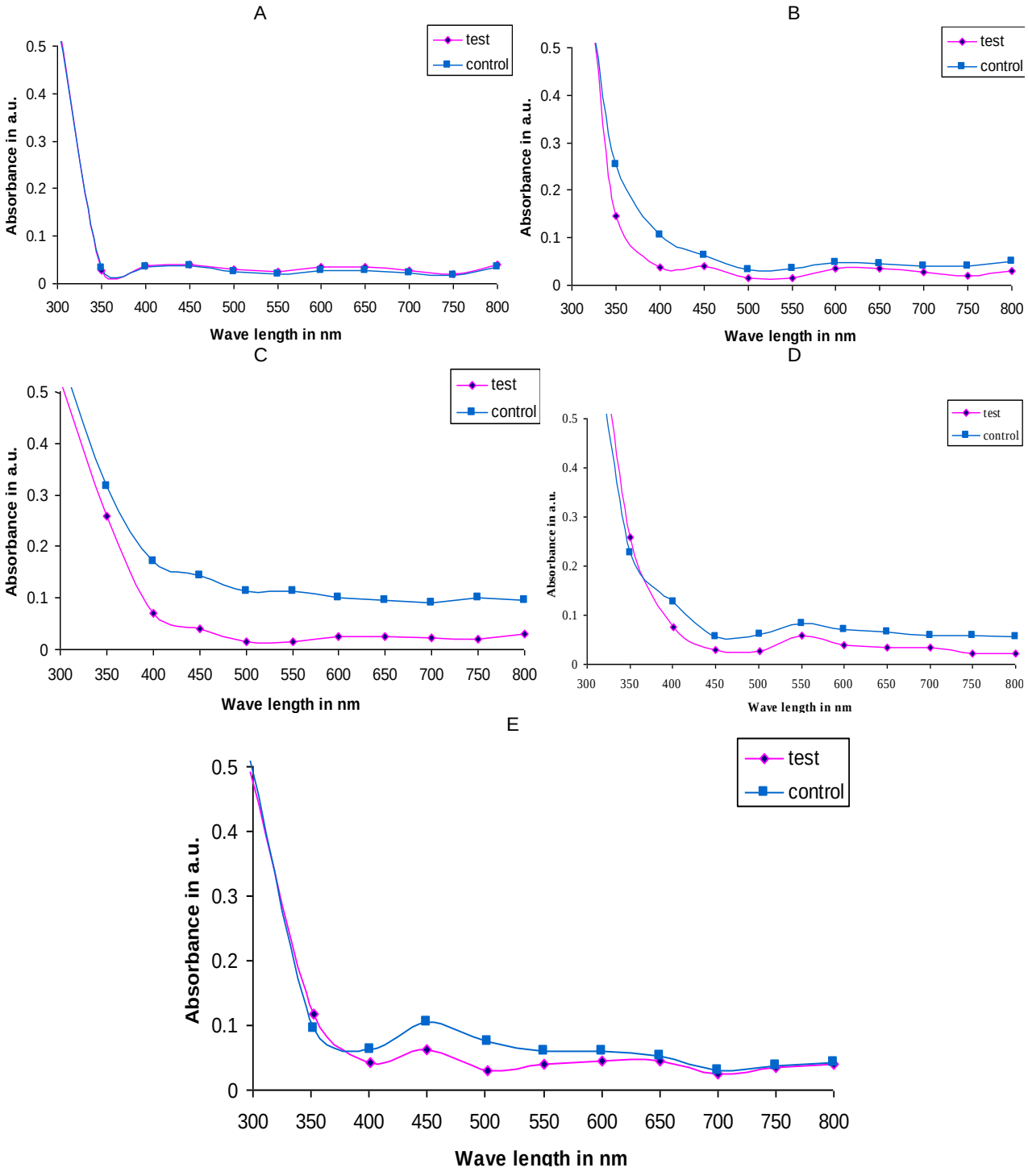

Figure 2. U. V. visible absorbance of $HAuCl_4$ various concentration treated *S. hygroscopicus* biofilm after 72 h. (A = with 10^{-1} mM, B = with 10^{-2} mM, C = with 10^{-3} mM, D = with 10^{-4} mM and E = with 10^{-5} mM).

shape and monodispersed (Figure 5). The characterization of $HAuCl_4$ treated actinomycetal biomass after 72 h exposure was carried out by XRD and metal nanoparticles size was determined. The XRD pattern of the $HAuCl_4$ treated sample (Figure 6) corresponds to that of pure gold nanoparticles. The size gold nanoparticles was ranging from 15 to 30 nm in diameter.

Present investigation reports actinomycete *S. hygroscopicus* have ability to synthesize good dispersed gold nanoparticles. The results agree with those reports

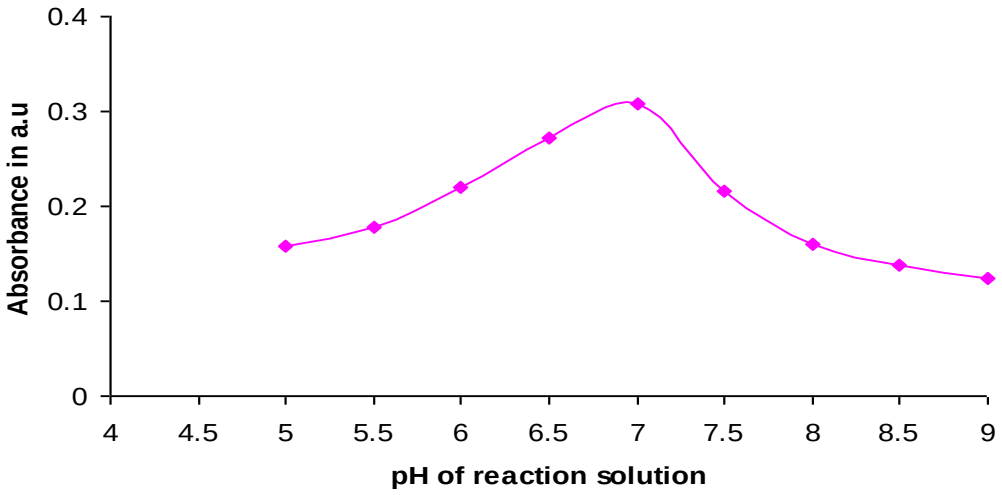

Figure 3. UV visible absorbance (550 nm) of $HAuCl_4$ (10^{-3} mM) treated *S. hygroscopicus* biofilm at various pH after 72 h.

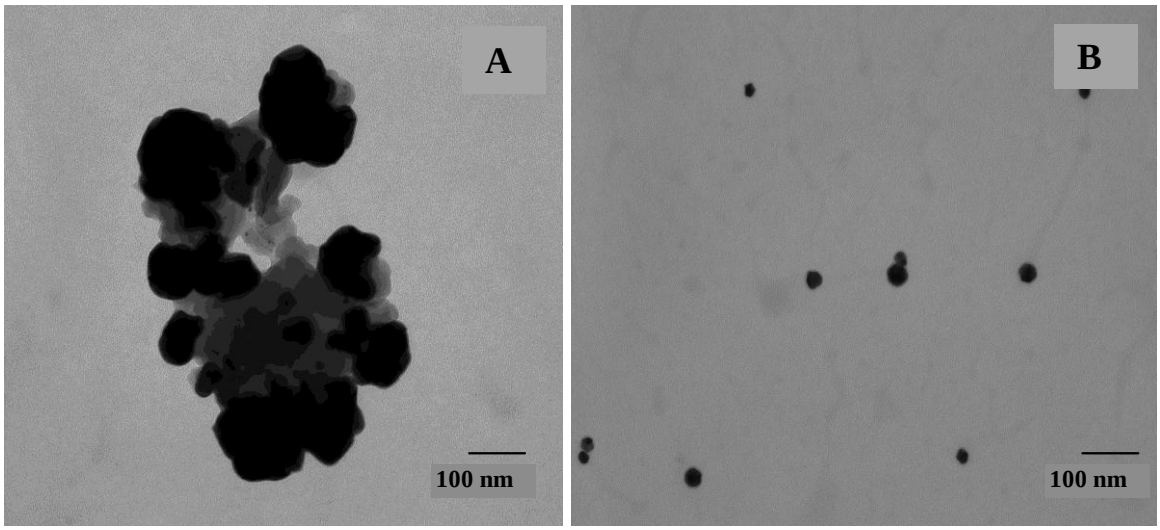

Figure 4. Characterization of nanoparticles by transmission electron microscopy: TEM image showing the gold crystals (A) before exposure to 10^{-4} aqueous solution $HAuCl_4$. (B) showing gold nanoparticles (after exposure to 10^{-4} aqueous solution $HAuCl_4$), synthesized by using *S. hygroscopicus*.

for gold nanoparticles synthesized by different methods (Ahmad et al., 2003; Shankar et al., 2003; Mukherjee et al., 2001; Nair and Pradeep, 2002). The size of gold nanoparticles using Scherrer equation was also determined by different research workers (Mukherjee et al., 2001). Silver nanoparticles have been characterized using XRD by various investigators (Balaji et al., 2009). The size of silver nanoparticles can also be determined by laser diffraction (Sadowski, 2008). X-ray diffraction (XRD), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were applied for the characterization of the gold nanoparticles assisted by bacterium *E. coli* (Liangwei, 2007). The synthesis of intracellular nanoparticles was studied by Mukherjee et al. (2001) by giving evidence of intracellular generation of gold nanoparticles provided by X-ray diffraction analysis of *Verticillium* biofilm deposited on Si substrate.

Conclusion

Gold nanoparticles are synthesized by the biomass of the bacterium, *S. hygroscopicus.* This is an economical, efficient, eco-friendly and simple process of biosynthesis of nanoparticles. The effect on pH and metal concentration of the synthesis of gold nanoparticles is studied.

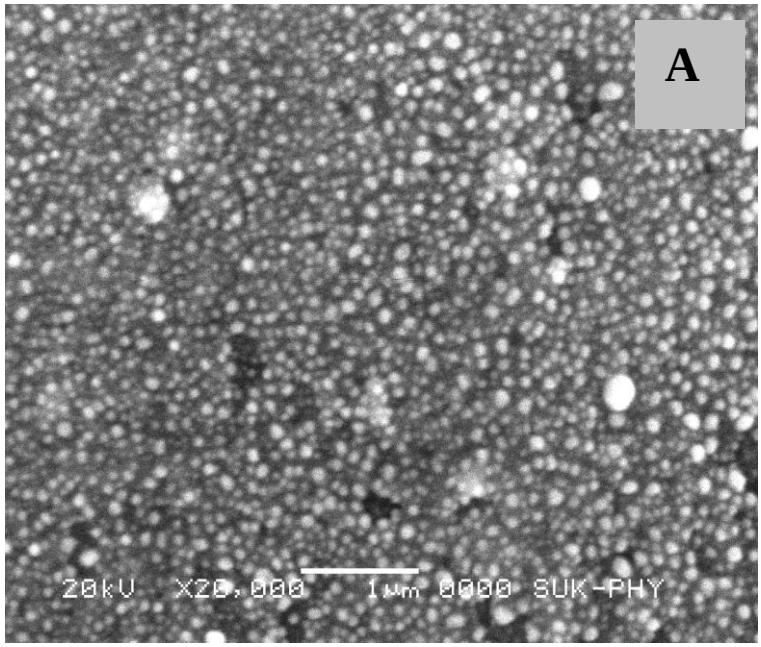

Figure 5. SEM image showing purified gold nanoparticles synthesized by *S. hygroscopicus.*

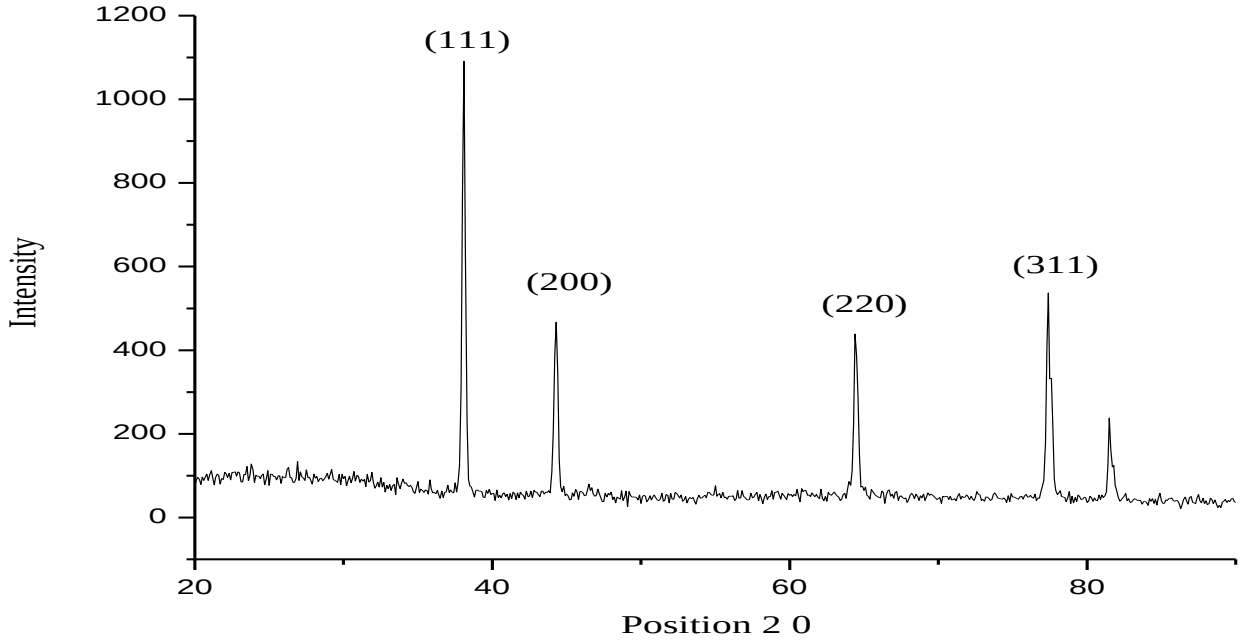

Figure 6. X-ray diffraction pattern of the gold nanoparticles.

The metal nanoparticles are partially purified by ignition method.

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