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Biosynthesis of Gold Nanoparticle using Cell-free Extract of Clinical Isolates *Staphylococcus Aureus* and *Escherichia Coli*

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ABSTRACT

Present study reports on biological synthesis of gold nanoparticles using cell free extract of *Staphylococcus aureus* and *Escherichia coli.* The cold extract is allowed to react with both 1mM and 10 mM solutions of $HAuCl_4$ which showed a colour change from yellow to dark cherry red after 1 hr. Gold nanoparticle formation is confirmed with UV-Visible spectrophotometer at 547nm.The 10mM concentration is found to be better for synthesis of more NPs using *E. coli.* XRD pattern exhibited 2θ values38.18°, 44.39°, 64.58°, 77.55° of 2θ value, corresponding to Bragg's diffraction at 111, 200, 220 and 311 plane of lattice structure closely matching with the standard values given in ICDD reference file. SEM analysis revealed morphological characteristics of nanoparticles of different sizes ranging from 70 to 200 nm. Thus, the present study throws new light on the suitability of *E. coli* as an alternative for conventional methods of chemical synthesis of gold nanoparticles.

Key words: Biosynthesis-gold, Nanoparticles-bacterial extract, Staphylococcus Aureus, Escherichia Coli

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INTRODUCTION

Nanoscience and nanotechnology has attracted a great interest over the last few years due to its application in many scientific areas such as energy, medical field, electronics, space industries etc. This interesting field of science deals with small structures and small-sized materials of dimensions in the range of few nanometers to less than 100 nanometers. These nanoparticles (NPs) show unique and considerably changed chemical, physical and biological properties compared to bulk of the same chemical composition, due to their high surface-to-volume ratio (Iravani, 2014).

Nonetheless, the biological synthesis is economic, nontoxic and environmental friendly. Unusual, but beneficial physical and chemical properties of nanoparticles lead to their wide range of applications in biotechnology, eco-remediation, catalysis, pharmacy and medicines, optics and electronics (Cao *et al.*, 2001; Hayward *et al.*, 2000; Pradhan *et al.*, 2001 and 2002; Zheng *et al.*, 2007; Dahl *et al.*, 2007; Hutchinson, 2008). The first evidence of bacteria synthesizing metal (silver) nanoparticles was established using the *Pseudomonas stutzeri* AG259 strain that was isolated from silver mine (Labrenz *et al.*, 2000). However, in the recent times, biosynthesis of nanoparticles using bacteria has emerged as a rapidly developing research area in green nanotechnology across the globe. Arya (2010) reported that many micro-organisms have the ability to produce inorganic nanostructures and metal nanoparticles with similar properties to chemically-synthesized materials. The microbes like bacteria, fungi, yeast, algae, higher plant etc. are the nanofactories for production of various metal NPs. To be precise, there are only few bacterial strains that have been tried so farfor synthesizing gold nanoparticles extra-cellularly viz. *Bacillus megatherium* (Wen *et al.*,2009); *B.subtilis* (Southam & Beveridge, 1994); *Desulphuriumdesulfuricans*, *Pyrobaculumislandicum* DSM 4184 and *Geovibrioferrireducens* (Kashefi, 2001); *E. coli* DH5co (Du *et al.*,2007); *E. coli* MC4100 (Deplanche & Macaskie, 2008); *Plectoneumboryanum* UTX485

(Lengke *et al*., 2006a; Lengke *et al*., 2006b); *Pseudomonas aerugenosa* (Husseiny *et al*., 2007); *Shewanella* algae strain-isolated from alga BRY (Kashefi *et al*., 2001); *Rhodopseudomonascapsulata* (He *et al*., 2008); *Stenotrophomonasmaltophilia* (Nangia *et al*., 2009) and *Pseudomonas fluroscence* (Rajasree & Suman, 2012).

Ahmad *et al*., (2003) demonstrated bacterial synthesis of monodispersed GNPs with extremophilic *Thermomonospora* sp. biomass via reduction of auric chloride ions ($AuCl_4^-$) through enzymatic processes. Konishi *et al*., (2004) reported GNPs synthesis using the mesophilic bacterium *Shewanella*, where H_2 is acting as an electron donor. He *et al*., (2008) and Shiying *et al*., (2007) revealed that the bacterium R*hodopseudomonas capsulata* produced spherical GNPs in the range of 10-20 nm, upon incubation of bacterial biomass with aqueous chlorauric acid ($HAuCl_4$) solution at a pH range of 4.0-7.0 upon 48 h of incubation. Bacterial cell supernatant of *Pseudomonas aeruginosa* has been used for reduction of gold ions and for extracellular biosynthesis of GNPs. *Bacillus subtilis* 168 has been reported to reduce water-soluble Au^{3+} ions to Au^0 and produce nanoparticles of octahedral morphology and dimensions of 5-25 nm inside cell walls.

In view of importance of biosynthesis of gold nanoparticles using microbes, presently an attempt is made to synthesis gold nanoparticles using Hydrogen Auric Chloride as metal source and cell-free extract of gram positive and gram negative bacteria viz. *Staphyloccocusaureus* and *E.coli* respectively.

MATERIALS AND METHODS

Preparation of HAuCl4 Solution

A metal source is required for the synthesis of nanoparticles. For synthesis of gold nanoparticles, hydrogen auric chloride ($HAuCl_4$) was chosen as metal salt. Hydrogen auric chloride trihydrate was purchased from HiMedia, Mumbai, India. Using double distilled water 500ml each of 1mM and 10mM solutions were prepared separately and the flasks were shaken well enough to dissolve the salt. The prepared solutions (1mM and 10mM) for the synthesis were stored at 4°C temperature in a refrigerator.

Preparation of extract of bacteria by cold process and synthesis of nanoparticles

Extracellular synthesis of silver nanoparticles method described by *Shahverdi et al., (2007)* was adopted with slight modifications. For the synthesis of gold nanoparticles, 100 mlCAS broth was prepared in flask, sterilized and inoculated with fresh growth of *Staphylococcus aureus and E. coli* obtained from the approved clinical diagnostic laboratory in Pondicherry. The inoculated flasks were incubated at 30°C for 72 hours. The culture was then centrifuged at 12,000 rpm for 15 minutes and the supernatant was discarded. Bacterial cells settled at the bottom. These were then washed 2-3 times in sterile distilled water to remove media traces. Afterwards, the bacterial cells were suspended in sterile distilled water and incubated for 48 hours. At the end of 48 hrs, suspension was centrifuged at 12,000 rpm and cell-free filtrate which contains osmotically lysed bacterial cell content was treated with 1mM and 10mM gold chloride and kept undisturbed for 48 hours at normal room temperature.

Detection of gold nanoparticles formation

After the 48 hrs cell-free filtrate and gold chloride (mixture), the preliminary detection of gold nanoparticles was carried out by visual observation of color change in filtrate. All the above reaction mixtures were then subjected to UV-vis Spectrophotometer analysis (Shimadzu UV-1700, Japan). The spectrum was scanned from 200-800 wavelengths at 1 nm resolution.

Characterization of gold nanoparticles

The morphology and nature (mono-dispersed or aggregated) of the nanoparticles were examined by SEM.

XRD Analysis

The gold nanoparticle solution obtained was purified by repeated centrifugation at 10,000 rpm for 30 minutes. For XRD analysis (Rigaku Ultima IV), the liquid phase nanoparticle solution was dried in an oven at 60°C and then ina muffle furnace at 750°C to form a powder. The dried powder was collected for determination of the formation of gold nanoparticles by an X'pert Pro x-ray diffractormeter operating at 40 kV and a current of 30mA with Cu Ka radiation in θ-2θ configuration.

SEM images

SEM samples of the aqueous suspension of gold nanoparticles were fabricated by dropping the suspension onto clean electric Stubs and allowing water to completely evaporate. SEM images were obtained on a Hitachi, Model: S-3400N Electron microscope.

RESULTS

Synthesis of Gold Nanoparticles:

Figure 1: Harvested bacterial isolates *(S.aureus)* and Harvested bacterial isolates (*E.coli)*

Figure 2: Reaction mixtures (1mM and 10mM of gold salt solution): Right set (*E. coli*) and left set (S. *aureus*).

Colour Change

Phase 1:

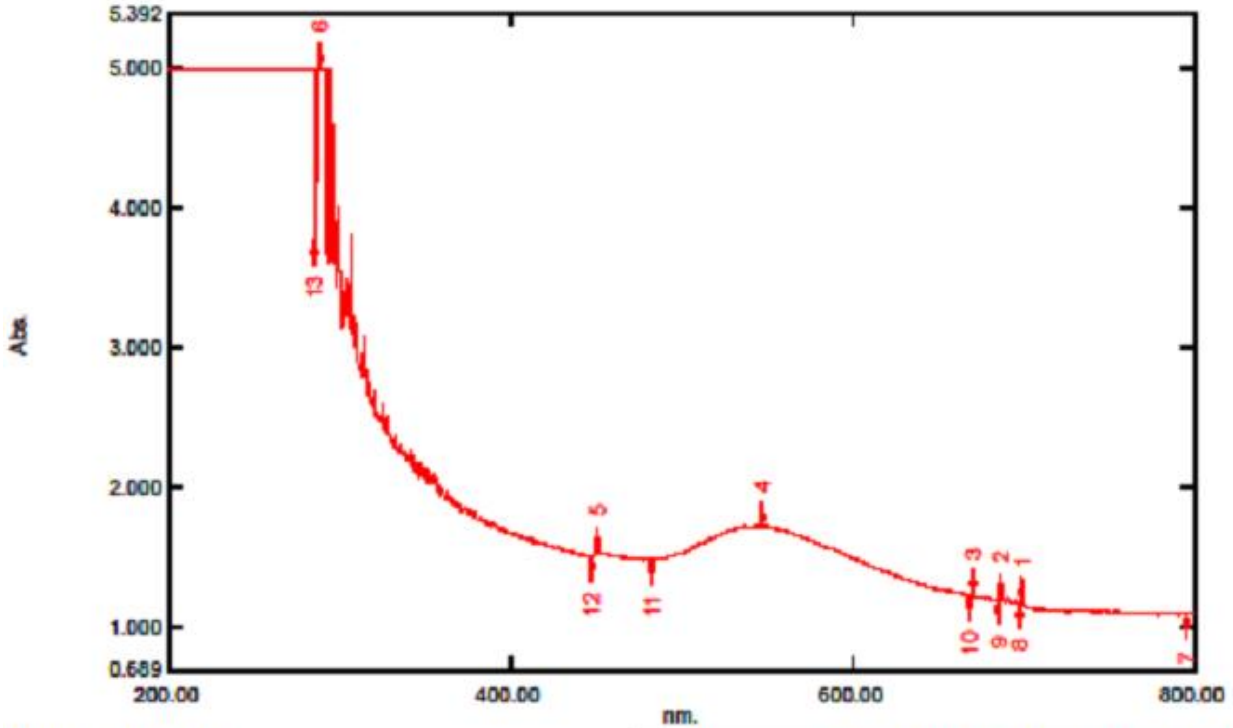
First part of the experiment was conducted to find out optimum metal concentration that could produce more NPs for given volume of bacterial extracts. Appearance of dark cherry red colour in the reaction tubes indicates the formation of gold nanoparticels and UV-Spectrum analysis revealed that 10mM concentration may be preferred for production of higher amount of NPs at a faster rate i.e. within 20 minutes of inoculation of *E.coli* than in P.aureus (Fig.1) and hence further steps to synthesis more NPs is done with extract of E.coli alone.

UV- Visible Spectroscopy:

Silver nanostructure exhibits interesting optical properties directly related to surface plasmon resonance (SPR), which is highly dependent on the morphology of the samples and the most important feature in optical absorbance spectra of metal nanoparticles. SPR is due to collective electron oscillation around the surface mode of the particles. As a function of chemical characteristics of solution, silver exhibits cherry red colour due to the excitations of their Surface Plasmon Resonance (SPR) when dissolved in water. Presently, the colour of the reaction mixture slowly turned into cherry red; a dark colour indicates the higher amount as well as smaller sized NPs. Appearance of cherry red itself indicate the formation of silver due to the reduction of gold chloride solution in the mixture by the extract of bacterial species used. The characteristic peak for gold ranges between 480 and 560nm and the variation from 480-560nm is due to various optical factors and the morphology of the particles present. The maximum peak obtained for the biosynthesized gold nanoparticles in the present study was at 547 nm with a highest absorbance of 1.723 for 10 mM concentration (*E.coli*) (Fig. 4) and lowest absorbance of 0.994 at 541 nm for 1 mM concentration (Fig. 3) and hence extract of E.coli is used with 10mM gold solution in the second phase to synthesis more of NPs for further particle characterization.

Figure 3: UV-Visible Spectra of reduction of gold salt to gold nanoparticles (10mM solution) by cell-free extract of *E.coli*

Data Set: saran_151816 - RawData



Measurement Properties

Wavelength Range (nm.):	200.00 to 800.00
Scan Speed:	Medium
Sampling Interval:	0.5
Auto Sampling Interval:	Enabled
Scan Mode:	Auto

Instrument Properties

Instrument Type:	UV-2400PC Series
Measuring Mode:	Absorbance
Slit Width:	0.1 nm
Light Source Change Wavelength:	360.0 nm
S/R Exchange:	Normal

Attachment Properties

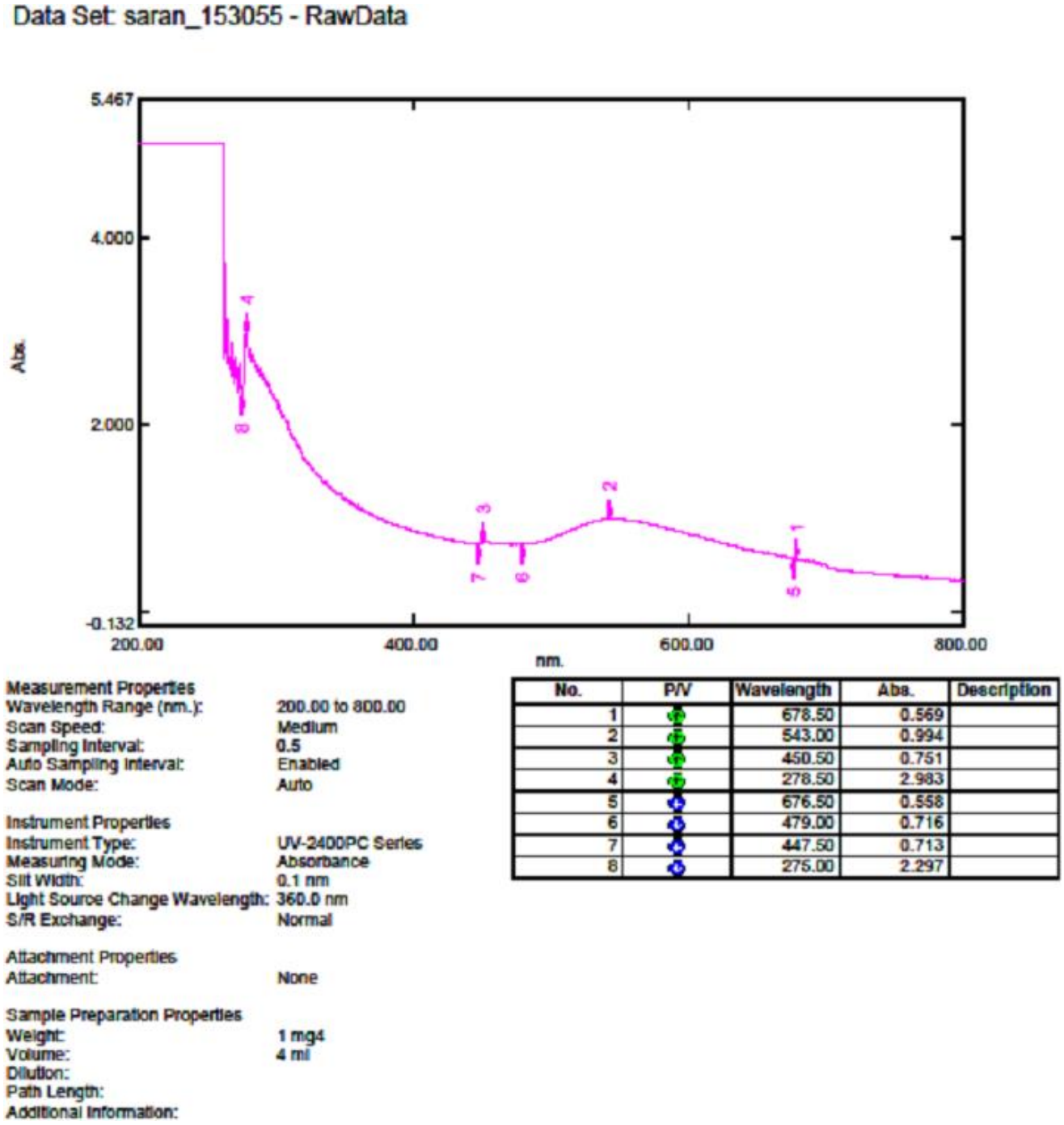
Attachment:	None

Sample Preparation Properties

Weight:	1 mg4
Volume:	4 ml
Dilution:	
Path Length:	
Additional Information:	

No.	P/V	Wavelength	Abs.	Description
1		699.50	1.178	
2		687.00	1.200	
3		670.50	1.229	
4		547.00	1.725	
5		451.00	1.532	
6		288.00	5.000	
7		795.50	1.081	
8		697.50	1.156	
9		685.00	1.183	
10		668.00	1.217	
11		482.50	1.479	
12		447.50	1.494	
13		285.50	3.757	

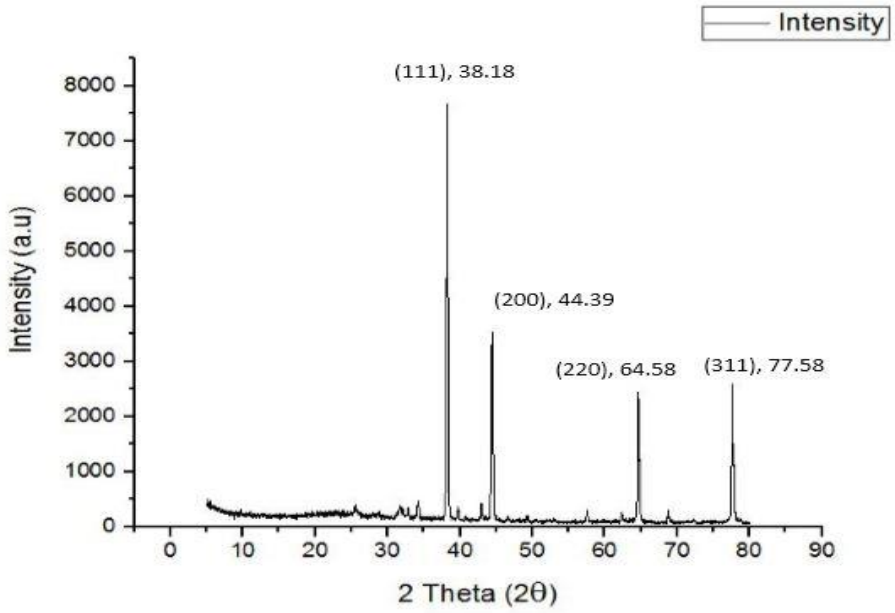
Figure 4: UV-Visible Spectra of reduction of gold salt to gold nanoparticles (1mM solution) by cell free extract of *Ecoli*



XRD Analysis:

X-ray diffraction pattern indicated the crystalline structure of the gold nanoparticles as depicted in fig 4.2. The present peak values are obtained at 38.18°, 44.39°, 64.58°, 77.55° of 2θ value, corresponding to Bragg's diffraction at 111, 200, 220 and 311 plane of lattice structure. They indicate the presence of gold nanoparticles and the maximum peak was obtained at 38.18 degree. Also, by the application of Scherer's equation the particle crystallite size was found to be 43.92nm at 111 lattice structure.

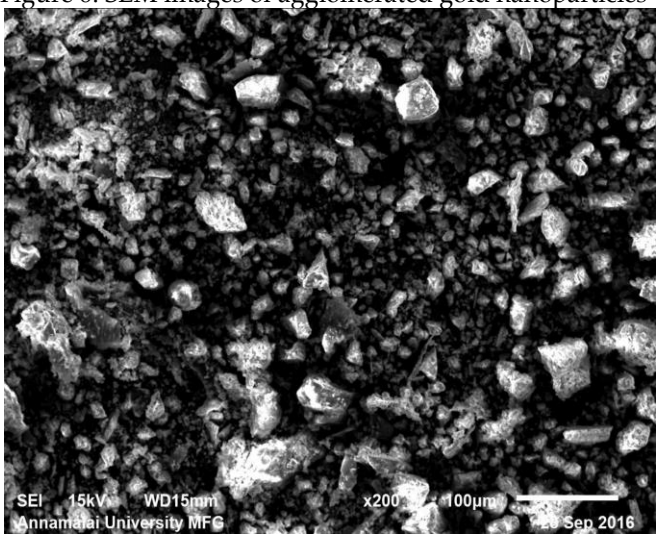
Figure 5: XRD Pattern of gold nanoparticles obtained for NPs synthesized using *E.coli*



SEM results

The scanning electron microscopic (SEM) images indicated the clustered nature of the particles and the surfaces of the aggregates are rough but are more or less spherical in size ranges from 70 - 160nm (fig. 6).

Figure 6: SEM images of agglomerated gold nanoparticles



DISCUSSION

The biological method for the synthesis of nanoparticles employs use of biological agents like bacteria, fungi, actinomycetes, yeast, algae and plants; thereby providing a wide range of resources for the synthesis of nanoparticles. In the present study, two bacterial species viz. *Staphylococcus aureus* (gram positive) and *E.coli* (gram negative) have been used to synthesis gold nanoparticles and the bacteria with higher efficiency to reduce metal salt, is arrived at.

The study involved two phases: During phase I, the highly efficient bacterial species was found out as *E.coli* which showed higher efficiency both in 1mM and 10mM metal concentrations based on the intensity of colour change and in terms of UV absorption level. Further, in the Phase II to produce higher amounts of nanoparticles required for further characterization, the experimental design included 250ml of 10mM solutions inoculated with *E.coli*. The change in colour from pale yellow to dark cherry red is reported to be the sign of formation of gold nanoparticles by the reduction of metal salt by the chemical constituent present in the cell free extract; similar colour changesis in close conformity with the reports of Suman *et al.* (2014), Singh *et al.* (2012), Sujitha and Kannan (2013), Boruah *et al.* (2012) and Huang *et al.* (2007).

The UV-spectrum indicated the presence of gold nanoparticles by showing characteristics peak between 542nm and 551 nm. It is reported that such deviation from the specific wavelength depends on the particle size and shape formed (Stephan & Macnaughtont, 1999). Huang et al., (2007) reported a peak at 570nm stabilized at 520nm. Suman *et al.* (2014) attributed 540nm to the surface plasmon resonance band (SPR) of the gold nanoparticles. Singh *et al.* (2012) reported that the maximum absorption for gold nanoparticles was observed at 545 nm. The usual peak in the case of gold nanoparticles is at 520 nm. When particles deviate from the spherical geometry, i.e. aggregation of gold nanoparticles in the solution begins to take place, the absorption appears in the long wavelength region as noticed in the present study.

The structural character of the nanoparticles formed has been subjected to XRD analysis and a typical XRD pattern was obtained as shown in figure 6.The 2θ values obtained are 38.18, 44.39, 64.58, 77.55, 81.72, 98.13, 110.80, 115.26, 135.42 for plane indices for 111, 200, 220, 311, 222, 400, 311, 420, 422 respectively and it is matched with the data sheet published by (ICDD) File no. 00-004-0784 on powder diffraction of gold nanoparticles. Similar 2θ values were also obtained for gold nanoparticles by Shankar *et al.,* (2004) and Suman *et al.,* (2014). The SEM image was compared with the UV-Vis spectrum and it is arrived at that the gold nanoparticles were in the size of 70 to 200 nm. Such a wide variations in the size of nanoparticles might be due to aggregation of gold nanoparticles formed due to the chemical and also due to the type of phytochemical constituent present in the bacterial extract. Similar size ranges have been obtained by Huang *et al.,* (2007), Ali *et al*., (2011), Suman *et al*., (2013) and Arunachalam and Annamalai (2013).

The causative factor behind such nanoparticle production using microbes, is basically reported to be nitrate reductase-mediated synthesis. A number of researchers supported nitrate reductaseas as the principal factor for extracellular synthesis of nanoparticles. To quote: extracellular synthesis of GNPs using *Rhodopseudomonascapsulata* carried out by He et.al, (2007), the bacterium *R. capsulata* is known to secrete co-factor NADH and NADH-dependent enzymes. The bioreduction of gold ions was found to be initiated by the electron transfer from the NADH by NADH-dependent reductase as electron carrier. Next, the gold ions (Au^{3+}) obtained electrons and are reduced to elemental gold (Au^0) and hence result in the formation of GNPs. Nangia *et al*., (2009) proposed the synthesis of GNPs by bacterium *Stenotrophomonasmaltophilia* and suggested that the biosynthesis of GNPs and their stabilization via charge capping in *S. maltophilia* involved NADPH-dependent reductase enzyme which converts Au^{3+} to Au^0 through electron shuttle enzymatic metal reduction process. Rai *et al.,* (2009) also reinforced the fact that biological agents secrete a large amount of enzymes, which are capable of hydrolysing metals and thus bring about enzymatic reduction of metal ions.

Works of Yong *et al.,* (2002) and Kalishwaralal et al., (2009) also revealed that the enzyme converts nitrate into nitrite and during the reduction, the electron is transferred to the silver ion; hence, the silver ion is reduced to silver (Ag+ to Ag^0). *Bacillus licheniformis* is known to secrete NADPH and NADPH-dependent enzymes like nitrate reductase that effectively converts Ag+ to Ag (Kalishwralal *et al.,* 2009). On the other hand, to confirm the reductase-mediated metal salt reduction, Kashefi *et al.,* (2001) made an attempt where purified nitrate reductase from *Fusariumoxysporum* was allowed to reduce silver nitrate along with NADPH in a test tube and the change in the color of the reaction mixture to brown and further analysis confirmed the silver nitrate reduction. It is also stated that a variety of secondary metabolites of various bacteria are also reported to be capable of reducing salts of silver and gold. Further, metabolites of some the microbes are also produce substances capable of reducing metal salts into their nanoparticles. For instance, metabolites like a ferric ion chelating compound siderophore present in bacteria ; protein hydrolyzing enzyme protease from Actinobacter spp; two types of siderophores, pyochelin and pyoverdin (Chrosa and Walsh, 2002); structurally, pyocyanin is 1-hydroxymethyl-5-methyl phenazyne, synthesized in the late logarithmic or stationary phase of the bacterial life cycle (Frank and De Moss, 1959); moreover many micro-organisms are reported to synthesize extracellularly protein hydrolyzing enzymes known as proteases which includes subclasses; cysteine, serine, aspartic and metallo-proteases depending on the amino acid residues present in bacterial cell (Rao et al 1998).

Therefore, it is reported from the present experimental study that bacteria could be used as better route for extra-cellular synthesis of gold nanoparticles; in particular E.coli. This green chemistry approach has many advantages as it is totally a cold process (at room temperature), no use of organic or inorganic chemicals except for the metal salt as the source of metal;, easily adoptable procedure which can be scaled up further to produce cost effective and eco-friendly NPs. It is also suggested that certain microbes are capable of producing specific type of enzymes extra cellularly and this trait can be prodently exploited to in the laboratory but mass culture of those bacterial species and tried for nanoparticle synthesis in future without totally lysing the bacteria to draw out the bacterial cell content as done in the present study.

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